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HAPLOID PLANT REGENERATION THROUGH ANDROGENESIS IN OILSEED CROPS: A REVIEW

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ABSTRACT

Anther culture is the process of induction and regeneration of plants from haploid male gametic cells with the aim of haploid and double haploid production. Due to its high efficacy and applicability in many plant species, it has exceptional potential for plant breeding and commercial exploitation of doubled haploids (DH). For development of F_1 hybrids, large number of homozygous plants as parental inbred lines, need to be generated in large scale. However, the production of homozygous lines by conventional method is time-consuming and also tedious process. Thus, the technique which reduces the time and labour for production of homozygous lines is required. *In vitro* techniques, such as anther culture can be utilized for development of haploids and further doubling the chromosome number can be used as parental inbred for development of F_1 hybrids. This review article deals with the current status of knowledge on the production of haploids and DHs through anther culture in some of the major oilseed crops.

Key words : Anther culture, Haploids, Double haploids.

Introduction

Haploid plant production is of great importance to shorten the breeding period in plant breeding programs. Obtaining pure lines in plant growing programs require an intensive work with huge labor and time. Obtaining one hundred per cent homozygous pure lines is a key point for the improvement and development of new cultivars. Haploid plants with a single set of homozygous chromosomes have become a valuable tool in plant breeding. Dihaploid plants that are homozygous at all loci with doubling of their chromosomes can be propagated by seed and reach full homozygosity in a single generation. Traditional methods take seven years to reach homozygosity. Dihaploidization methods provide significant advantages in terms of gaining homozygosity in a short period of one year and bringing pure lines into agriculture. Anther culture and irradiated pollen technique are among the most widely used techniques in this respect; where physical or chemical agents are used to induce mutated pollen grains and anthers that are subsequently employed to develop dihaploids through *in vitro* cultures. These techniques are a good source to facilitate gene mapping, cytogenetic research, and evolutionary studies. Irradiated pollen culture techniques have been applied to many oilseed crops to obtain pure lines. This study highlights some salient features of producing dihaploids using irradiated pollen grains and their maintenance.

Haploid is a general term that refers to a plant containing the gametophytic number of chromosomes, that is, a single set of chromosomes in the sporophyte. Although in some special cases individual workers obtained haploids spontaneously or experimentally by special methods, until 1964 the large-scale production of haploids in higher plants was only a theoretical possibility. Guha and Maheshwari (1964) reported direct development of embryos from microspores of *Datura innoxia* by the culture of excised anthers. Basically, this technique involves the isolation and sterilization of flower buds, aseptic removal of anthers with subsequent culture either on agar-solidified medium or in liquid medium a

tissue culture room. The development of this technique for the induction of androgenesis by the culture of excised anthers played a very important role in the resurgence of interest in haploids.

Haploids derived from anther and microspore culture have considerable potential for plant breeding because of the time saved by the reduction of the classical selection cycle period and the genetic value of isogenic lines or homozygous diploids. Also, the presence of a single set of chromosomes allows the detection of mutations controlled by recessive genes.

This review deals with the main aspects of haploid production through *in vitro* anther culture in some of the major oilseed crops like sunflower, safflower, linseed, Niger and *Brassica* species.

Bud size and microspore stage

Haploid breeding is a very desirable way to select homozygous lines within a shorter time period. Determination of capitula (buds) stage is very important since it facilitates the collection of the best responsive anthers. Investigating the precise stage of the microspore development in order to promote efficient embryo production through anther culture is critical (Garkusha *et al*., 2017). Currently, there is valid evidence to suggest that the age of the anthers (and correspondingly, the developmental stage of the contained microspores) plays an important role in the successful production of haploid plants. Most researchers recommend using immature anthers carrying medium to late uninucleate microspores. Hence, the most suitable material for anther culture is the selection of main capitula (buds), which are emerging from flowering branches (Murthy *et al*., 2000). The stage of pollen development is usually tested in one anther per floral bud size by the acetic-carmine method (Sharma and Sharma 1972). The anthers are collected from flower buds at different stages of development and squashed in acetocarmine staining solution (1% acetocarmine in 45% acetic acid) for observation under an optical microscope to determine the stage of pollen development. DAPI (40, 6-diamidino-2-phenylindole dihydrochloride) fluorescent staining has also been used.

The pollen development stage is a complex factor that strongly affects the success of anther culture. In sunflower, Saensee *et al*. (2018) reported that disk floret length or anther size, is a better predictor of microspore developmental stage and they established significant positive correlations between disk floret length or anther size in terms of anther length and anther width with the higher percentage of mid-to late- uninucleate microspores that responded well and produced embryogenic callus in the optimum culture medium. Similarly, the microspores in the sunflower at the mid-to-late mononuclear stage were found to more responsive to anther culture (Garkusha *et al*., 2017). Nurhidayah *et al*. (1996) achieved the best results with anthers of distant hybrids that carried early uninucleate microspores. However, Gurel *et al*. (1990) reported a high rate of callus formation and shoot organogenesis using anthers with the most microspores at the tetrad stage compared with anthers containing uninucleated microspores. It should be noted that for the induction of the morphogenesis of some genotypes, it was critically important that the microspores were in the tetrad stage. Several researchers have shown that the stage between the dyad and tetrad is the best for subsequent callus formation and direct embryogenesis. Several researchers; Saensee *et al*. (2018) and Prabakaran *et al*. (2000) have proposed selecting sunflower inflorescences at the R-5.1 stage of development, according to the classification by Schneiter and Miller (1981). In accordance with these results, anthers isolated from the flower buds in the tetrad stage of meiosis or the uninucleate microspore stage induced callus in sunflower (Gurrel *et al*., 1990).

The developmental window of embryogenic competence differs depending on the species tested but, generally, uninucleate stage of microspore found to more responsive to anther culture in many of the species. In line with this, in safflower, Nakas *et al*. (2023) reported that immature capitules with white flowers, which indicates the early uninucleate stage of anther had responsive microspores. Also, Kassa *et al*. (2024) used uninucleate stage of anthers for culture because microspores at this stage are advantageous for many genotypes and species in anther cultures. Typically, florets are white at the early-late uninucleate stage; florets become yellow as they mature, and the colour of the floret was also employed as an index to select the anthers with early-late uninucleate microspores.

In linseed, healthy and vigorously growing flower buds which were collected for culture four days before flowering when the microspores were estimated to be at the uninucleate stage (Kurt *et al*., 1998). Buds at the middle of late uninucleate stage of microspores are having length of 5-7 mm, width 1.5-3 mm, length of anther 1-2 mm. Generally, it was found that the most suitable bud length for the highest embryogenesis ranged from 5-6 mm for all genotypes used. Mikelsone *et al*. (2011) found that buds of width 3 mm are not suitable for anther culture establishment: anthers obtained from these buds did not form any embryos. Contrastingly, Burbulis *et al*. (2005) observed flower buds at 3.5-4.0 mm in length at mid

uninucleate size as determined by microscopic observations were more responsive to safflower anther culture. Uninucleate size (Obert *et al*., 2004) and late uninucleate size microspores (Nichterlein *et al*., 1991) were found to be better stage for callus induction in safflower. Hence, in safflower, early uninucleate to late uninucleate stage microspores based on different genotypes were used for callus induction by many researchers.

In Niger as in many other species, the uninucleate stage is the most responsive to anther culture. This stage of pollen grain can be determined by measuring the diameter of the flower bud (5.9 to 6.5 mm). The color of anthers corresponding to the mid to late uninucleate stage is generally whitish green and anthers with tetrads are of yellowish color (Adda *et al*., 1994). The size of capitula can also be correlated with the stage of microspores inside the anther. Murthy *et al*. (2000) reported that the young and immature capitula of 5-6 mm in diameter were having uninucleated microspores and these buds can be conveniently used for anther culture in Niger. Immature capitula with whitish green florets having microspores at mid to late uni-nucleate stage can also be used for anther culture (Hema and Murthy, 2007). Tesfaye *et al.* (2010) reported that a maximum number of responsive anthers for anther culture were present in light green or greenish yellow color disc florets. Sarvesh *et al*. (1993) also reported that the disc florets of light green color containing anthers were most responsive for callus induction. The colour of anthers corresponding to the uninucleate stage of pollen grains is whitish green and anthers with mature pollen are yellow in colour. Similarly, Maurya *et al*. (2024) observed that one week old buds carried most of the light green to greenish yellow color disc florets that had uninucleate microspores after flower initiation.

In *Brassica carinata*, Abraha *et al*. (2008) reported that sufficient number of embryos can be obtained from buds which are 2.5–3.5 mm long. These results are in accordance with experiments on *B. carinata* and other genotypes of the genus *Brassica*, where the size of buds is considered as an important factor affecting microspore embryogenesis, (Telmer *et al*., 1992 and Vincente and Dias, 1996). Chuong and Beversdorf (1985) recommended bud size of 2.5 to 3 mm in *B. carinata*. Thurling and Chay (1984) reported bud size from 2 to 3 mm for tested genotypes of *Brassica napus*. Lichter (1982) observed that the bud size with microspores in optimal stage for *Brassica napus* genotypes was between 3 to 4 mm.

Bud pretreatment

An important condition required for microspores to switch from a gametophytic to a sporophytic developmental pathway is the stress pretreatment of the plants (Thengane *et al*., 1994; Saji and Sujata, 1998; Garkusha *et al*., 2017) or flower buds (Catmak *et al*., 2019). Previously, it is reported by many researchers that anthers in uninucleate microspore stage need to be collected to induce embryogenesis in oilseed crops (Simarro and Neuz, 2007; Ibrahim *et al*., 2014; Mishra and Rao, 2016; Tripathy *et al*., 2019). Hence, the stress pretreatment of inflorescences or flower buds at a low positive temperature is the most efficient for any crop species.

In particular in sunflower, Garkusha *et al*. (2017) reported that before planting anthers on the nutrient media, the cut anthodia were incubated for 1 – 6 days at 10°C. Apart from this, many researchers had found positive results associated with the increase in temperature for pretreatment of anthers. In line with this, Gurrel *et al*. (1990) reported that best callus induced from anthers that were first kept at 30°C under light (400 lux) for three days and then maintained under a 16 hrs photoperiod with an illumination of 3,000 lux. Saensee *et al*. (2018) reported that flower buds at the R 5.1 stage which were collected before anther dehiscence then were kept in a cool block had better response to callus induction. Along with the pretreatment of anthers cultivation of the anthers in the dark until the development of callus seems to be a crucial factor favoring androgenesis in sunflower (Saji and Sujata, 1998). A pretreatment at 32 or 35ºC has been shown favorable for anther culture by Jonard and Mezzarobba (1990). But the studies of Coumans and Zhong (1995) showed that complete loss of microspore viability after three days of culture when precultured at 32ºC in the dark. Thengane *et al*. (1994) reported a stimulatory effect of cold pretreatment on embryo induction in sunflower and reported that the effect of cold treatment indirect on microspores.

In safflower, it has been found that pretreatment of either flower bud or anther significantly enhance the anther response and also, there found to be positive results when the anthers inoculated after pretreatment with the increase in frequency of callus induction over untreated capitula (Rajendra Prasad, 1991). However, the percentage of calli gradually decreased with prolonged periods of pretreatment and almost all the inoculated anthers lost their callusing ability after 15 days of pretreatment (Rajendra Prasad, 1991). Nakas *et al*. (2023) reported that a pre-treatment to capitules at 4°C for 3-4 days induced higher percentage of callus which

is line with the findings of Prasad *et al*. (1990).

In linseed also, for the pretreatment of flower buds the petri dishes were kept in the dark room at 4°C for three days (Kurt *et al*., 1998). The influence of cold treatment was observed by several authors (Pretova *et al*., 2006; Rutkowska-Krause *et al*., 2003 and Obert *et al*., 2004). Mostly used cold stress temperatures ranged from +4 °C till +8°C. All of authors mentioned influence of genotype and duration of the treatment on embryogenesis level (Mikelsone *et al*., 2011). To improve anther culture response of recalcitrant genotypes some physiological and environmental factors can be employed that can influence the response of anther culture (Chen and Dribnenki, 2002). Usually, a stress is provided by heat or cold treatment or by starvation to induce switch of developmental programme from pollen development to *in vitro* androgenesis. There are also better results in linseed anther culture due to pre-cultivation of the donor material at 8ºC for 7 days (Obert *et al*., 2004).

Similarly, in Niger, flower buds which are chilled for 24 hours at 4° ± 1°C in a refrigerator are found to give better results compared with the untreated ones (Adda *et al*., 1994). We can also induce embryogenic callus through anther culture in Niger with the pretreatment of capitula at 4ºC for one day prior to anther culture (Sarvesh *et al*., 1993). However, Murthy *et al*. (2000) reported that pretreatment of capitula at 4-6ºC for five days prior to anther culture, have responded better to the cultural regimes and developed embryos. Other than these, when the mouth of the beaker was tightly wrapped with aluminum foil to maintain a high humidity and kept in dark at 4°C for three days has found to give better results (Hema and Murthy, 2007). But, among all these a 24-h cold pre-treatment was reported to be the best, which was also confirmed by Maurya *et al*. (2024) in anther culture of Indian genotypes of Niger and Tesfaye *et al*. (2010), who reported that the best cold pre-treatment duration of capitula (buds) for better induction of callus in anther culture of the Niger was 24 hours.

For *Brassica* species, Alam *et al*. (2009) reported bud treatment at 5°C for 24 hours would give better results. The same conditions were reported by Sayem *et al*. (2010).

Culture medium

A pivotal role in the induction of microspore embryogenesis is played by the culture medium composition. The diverse genotypes show very different basal medium requirements to induce pollen-derived plant formation. Auxin and cytokinin PGRs are necessary components of any culture medium for the induction of morphogenesis. Among the various factors that influence the androgenesis, culture medium and/or its components are most important once. As has been studied by many scholars, callus formation definitely requires exogenous PGRs. The most commonly used basal media for anther culture are N_6 medium (Chu, 1978), MS medium (Murashige and Skoog, 1962), Nitsch and Nitsch (NN) (1969) medium and B_5 medium (Gamborg *et al*., 1968), Linsmaier and Skoog (LS) (1965) medium and many others.

In sunflower, Saensee *et al*. (2018) used MS medium modified with various hormones and additives and sucrose (30 g/l) to induce callus. To inoculate sunflower anthers, basal MS medium with agar has also been used, with varying qualitative and quantitative content of phytohormones á-Naphthaleneacetic acid (α-NAA) and 6-Benzylaminopurine (6-BAP) as well as $AgNO_3$ (Garkusha *et al*., 2017).

In safflower, among some of the commonly used media, the culture media N6 and Chaleff's had not shown any response in inducing calli. However, on LS medium callus initiation was found to be with low frequency and MS medium induced callusing with maximum response (Rajendra Prasad, 1991). Along with the basal media, callus can also be induced with the addition of some of the auxins and cytokinins. In accordance with this, Nakas *et al*. (2023) reported callus initiation from the safflower genotypes on BAP and NAA. However, Kassa *et al*. (2024) induced callus with different concentrations of TDZ and IBA (1.0 mg/l) and also reported that this hormonal combination can be the most effective treatment for eliminating the secretion of phenolic substances in safflower anther culture.

In linseed, the type of auxin used to induce callus appeared to determine the organogenetic capacity of anther culture-derived calli in linseed. The combination of 2,4-D (2.0 mg/1) with BAP (1.0 mg/l) in the induction medium dramatically improved organogenetic capacity of anther culture-derived calli and subsequently significantly increased the overall efficiency of regeneration in linseed compared with the combination of NAA (1.0 mg/1) and BAP (2.0 mg/1). But, Thiamin hydrochloride in the induction medium was not essential for callus induction and shoot regeneration from cultured anthers of linseed, suggesting that linseed microspores may be able to synthesize the vitamin *in vitro* (Chen *et al*., 1998). Most commonly used basal media for callus induction is MS media. However, many researchers have reported better results with use of modified MS media. Burbulis *et al*. (2011) was able to induce callus when the

anthers were inoculated onto a plastic petri dish containing 15 ml of modified MS induction (NH_4NO_3 165 mg/l) medium with different combinations of cytokinin and auxin (2.0 mg/l BAP + 1.0 mg/l NAA and 1.0 mg/l BAP + 2.0 mg/l IAA). Apart from using solid media for callus induction, Kurt *et al*. (1998) used liquid medium to induce callus in linseed anther culture and found that the rate of callus initiation was low and the cultivars significantly affected callus induction rate in linseed. High frequency of callus induction can also be achieved with the use of Nichterlein (NC) induction medium and modified NLN_{82} medium in linseed (Mikelsone *et al*., 2011). Other than these media, Mo, N_6 and NN media supplemented with various combinations of growth regulators have found to give better results with differential rate of callus initiation (Obert *et al*., 2004).

As in sunflower, safflower and linseed, we can induce callus in Niger with use of various media along with different growth regulators at optimum concentration. Anther in Niger was first time done by Sarvesh *et al*. (1993). They were able to induce callus on five basal media supplemented with 2,4-D (2.0 mg/l) + kinetin (0.3 mg/l) or NAA (2.0 mg/l) and kinetin (0.3 mg/l). Anthers inoculated on LS medium supplemented with kinetin (2.0 mg/l) or Gamborg's B_5 medium incorporated with NAA (2.0 mg/1) and kinetin (0.3 mg/1) have found to induce better percentage of callus (Adda *et al*., 1994). Murthy *et al*. (2000) also used B_5 medium but it was supplied with varied concentrations of 2,4-D and BAP for Niger anther culture. Induction of embryogenesis from cultured anthers and plantlet regeneration has also been reported in Niger (Murthy *et al*., 2000 and Sarvesh *et al*., 1993). However, the frequency of embryo production and plant regeneration was very low. Therefore, Hema and Murthy (2007) studied the effect of sugars on induction of embryogenesis and plant regeneration from the cultured anthers of Niger and reported that sucrose is crucial for callus induction. MS medium supplemented with 2,4-D (2.0 mg/l), the combination of 2,4-D (2.0 mg/l) and kinetin (0.3 mg/l), NAA (2.0 mg/l) and the combination of NAA (2.0 mg/l) and kinetin (0.3 mg/l) hormones was used for callus induction (Maurya *et al*., 2024).

For *Brassica* species, Alam *et al*. (2009) used MS medium supplemented with 1.0, 2.0 and 4.0 mg/l 2,4-D along with the constant addition of BAP (1.0 mg/l) for callus induction. Sayem *et al*. (2010) used ten combinations and concentrations of media, for callus induction in *Brassica* species.

Culture conditions

Anther cultures are usually incubated at 24–27ºC and exposed to light at an intensity of about 2,000 lux for 14 hrs per day (Reinert and Bajaj 1977), but other culture conditions have also been reported.

For example, in sunflower the cultures incubated at 25 ± 2ºC in dark conditions for 5 days and kept under 16 hrs illumination with 3000 lux light intensity found to induce better callus (Saensee *et al*., 2018). The same conditions were reported by Saji and Sujata (1998), but they incubated at 25±2ºC in dark in a growth room till callus induction.

In safflower, the cultures maintained under continuous light (2000-2500 lux), at 25ºC with 50- 60% humidity found to be the best cultural condition (Rajendra Prasad, 1991). Nakas *et al*. (2023) also applied similar conditions to cultures in which, initially kept in the dark at 25ºC with 50-60% humidity for 10 days and then continued the same culture condition with 16/8 day and night illumination. We can also induce callus with higher frequency by keeping the cultures in complete darkness at a temperature of 25 ±1ºC (Anelay *et al*., 2024).

In Linseed also cultures incubated at 25°C in the dark found to give better results for callus induction. The best conditions for cultures are 27/24°C (day/night) under a 16 h photoperiod, at a light density of 50 ìmol m^{-2} s^{-1} (Burbulis *et al*., 2011). A slight decrease in temperature had also given better results where anther cultures incubated at 23°C under a 16-h photoperiod with light source providing 8.6 Wm^{-2} light intensity (Obert *et al*., 2004).

In Niger, cultures incubated under continuous, cool white fluorescent light (2000 lux) at 26 ± 2°C (Adda *et al*., 1994). The cultures were incubated in dark for 10 days at 45°C. After that, the cultures were incubated under cool white fluorescent light with 16 hrs photoperiod (40 µmol m^{-2} s^{1}) and 60-65% relative humidity (Murthy *et al*., 2000). The cultures were incubated in dark at 24±2°C for 2 weeks and then at 24 ± 2°C and 16-h photoperiod with irradiance of 40 µmol m^{-2} s^{1} provided by cool white fluorescent tubes (Hema and Murthy, 2007). Each culture bottle was placed in a growth chamber maintained at 25 ± 2°C and 2000 lux light intensity for callus regeneration (Maurya *et al*., 2024). All the cultures were incubated under continuous white fluorescent light of 2000 lux at 26 ° ± 2°C (Sarvesh *et al*., 1993).

For *Brassica* species, Alam *et al*. (2009) incubated the culture vials at 25±1°C with 16 hrs photoperiod and Sayem *et al*. (2010) observed that 28°C room temperature for 3-4 weeks in complete dark is better for callus formation.

Callus formation

In Sunflower, **t**he MS medium supplemented with NAA (2.0 mg/l), BAP (1.00 mg/l) and coconut water (100 ml/l) produced the highest frequency of callus induction (60.44%) (Saensee *et al*., 2018). Optimum concentrations of 6-BAP for this hybrid were found to lay within the range from 0.5 mg/l to 1.0 mg/l. The increase in the 6-BAP concentration in the callus inducing nutrient medium up to 2.0 mg/l had a negative effect on callus genesis (Garkusha *et al*., 2017). Different variables using MS basal medium supplemented with NAA (2.0 mg/l) and N_6-benzyladenine (BA) (1.0 mg/l) have also been tested for their ability to enhance the frequency of anther callusing and subsequent embryogenesis.

In safflower, Rajendra Prasad (1991) reported 46 per cent of callus induction on MS medium supplied with BAP (3.0 mg/l) and NAA (0.5 mg/l). But as the concentration of BAP and NAA were increased it has found to affected the rate of regenerable callus adversely (Nakas *et al*., 2023). However, a slight decrease in concentration has found to give better results with NAA (0.1 mg/l) and BAP (0.5 mg/l) (Kassa *et al*., 2024). With some modifications in the cultural medium, Prasad *et al*. (1990) reported callus production of 59 per cent with BA (1.0 mg/l) + NAA (0.1 mg/l) + sucrose (3%). The highest callus formation frequency (46.20%) was observed in the Turkan genotype cultivated on MS medium containing TDZ (0.5 mg/l) and IBA (1.0 mg/l).

The rate of callus induction varies not only with the type of growth regulators and their concentration but also with the genotypes used for anther culture. In linseed, on MS medium supplied with BAP (2.0 mg/l) + NAA (1.0 mg/l) the mean values of the percentage of anthers producing callus ranged from 0% for the cultivar 'Zaltan-1' to 61.67% for the 'Szaphir' on the same medium supplemented with 6% sucrose (Burbulis *et al*. 2011). There can be differential rate of callus initiation based on the pretreatment of anthers. Kurt *et al*. (1998) reported 0 to 6.7% callus induction in solid medium with no pretreatment of anthers, from 0 to 2.0% in solid medium with pretreatment, 0 to 2% in liquid medium with no pretreatment and 0 to 4.7% in liquid medium with pretreatment. Better embryogenesis and plant-regenerants can be obtained on NLN82 induction medium (Mikelsone *et al*., 2011). The induction of callus formation from cultured anthers can also be done on N_6 (12%) and NN media (2.8%) (Obert *et al*., 2004).

The earlier studies on Niger anther culture by Sarvesh *et al*. (1993) reported induction of embryogenic and non embryogenic callus. Anthers cultured on LS medium supplemented with 2,4-D (2.0 mg/1) and kinetin (0.3 mg/l) have found to induce embryogenic callus and the anthers cultured on LS medium supplemented with BAP (1.0 mg/1) and kinetin (0.2 mg/1) produce non-embryogenic callus. Compared to the combination of NAA and kinetin, the combination of 2,4-D and kinetin was found to be more effective for callus induction (Maurya *et al*., 2024). Similar results were also reported by Sarvesh *et al*. (1993). Tesfaye *et al*. (2010) observed 40 to 80% callus induction frequency in NN medium and B_5 medium supplemented with kinetin (2.0 mg/l and 0.3 mg/l). Direct embryo induction can be achieved from anthers on B_5 medium supplemented with 2,4-D and BA combination with high levels of sucrose. These results reveal that 2,4-D is the essential component in the medium for induction of embryogenesis (Sarvesh *et al*., 1993). Similarly, there are various recent reports wherein the embryogenesis is induced from anthers, either in presence and 2.4-D alone or under the influence of both auxin and cytokinin (Murthy *et al*., 2000). Other than MS medium, LS medium containing 2,4-D also promotes embryogenic callus ranging from 30% (IGP-76) to 90% (Ootacamund) frequency and B5 medium supplemented with NAA and kinetin induces non-embryogenic callus from 19-65% (Adda *et al*., 1994). Of the different basal media that are commonly used, LS medium containing 2,4-D (2.0 mg/l) and kinetin (0.3 mg/l) is found to be superior followed by N_6, MS, B_5 and Chaleff's R-2 for callus induction from anthers of the Niger variety Ootacamund (Sarvesh *et al*., 1993). Among the 2,4-D and BA combination tested with various levels of sucrose (4- 8%), optimum response and embryo induction was noticed on medium supplemented with 2,4-D (2.0 µM) and BA (1.0 µM) with 6% sucrose. (Murthy *et al*., 2000). Along with the optimum concentration of growth regulators sucrose concentration also plays an important role in callus initiation. For instance, anthers cultured on B5 medium with 2,4-D and BA with 2% sucrose developed only callus, whereas, anthers cultured on medium with 2,4-D and BA with higher level of sucrose (4-8%) have found to induce embryos without intervening callus. This indicates that high level of sucrose along with growth regulators is essential for direct embryo induction from anthers of Niger (Murthy *et al*., 2000). Along with sucrose other sugars also found to induce callus with lesser frequency. Accordingly, in Niger anther cultures, maltose has shown poor response and only few concentrations (0.10 - 0.25 M) have induced embryogenesis in less per cent of anthers (Hema and Murthy, 2007).

For *Brassica* species, Alam *et al*. (2009) reported that the medium supplied with MS + 2,4- D (4.0 mg/l) +

BAP (l.0 mg/l) showed the best performance (52.50%) for callus induction. Similarly, Sayem *et al.* (2010) reported maximum rate of callus induction on MS + 2, 4-D (0.5 mg/l) in *Brassica* species. Nagoo *et al.* (2018) observed that the best callus development and proliferation was achieved in MS medium supplemented with 2,4-D (1.0 mg/l) and NAA (0.5 mg/l) in *Brassica rapa*.

Shoot formation

Shoot regeneration from anther culture derived callus is a critical phase of the whole androgenic process. One of the factors affecting the efficiency of regeneration from anther culture derived callus is the plant genotype and the crop species.

In safflower, Prasad *et al*. (1991) obtained the highest shoot regeneration from anthers of safflower genotypes in a combination of BAP (2.0 mg/l) and NAA (0.5 mg/l). A slight decrease in BAP and NAA had also given better results. Nakas *et al*. (2023) could able to get shoots from the application of BAP (1.0 mg/l) + NAA (0.1 mg/l). The variation in shoot regeneration between the genotypes may be associated with callus quality and its regeneration capacity. For instance, genotypes may produce poorer shoot regeneration due to friable calli whereas, compact calli induces better shoot regeneration (Nakas *et al*., 2023). This result is also in line with Mosoh *et al*. (2024), who found that more compact and greener calli promote shoot development and induced the highest number of shoots on their surface. Additionally, there were substantial differences in shoot regeneration associated with the BAP concentration. When considering shoot regeneration frequency among other parameters, the highest values of shoot regeneration frequency (16.99%) were seen at BAP (2.0 mg/l) + NAA (0.5 mg/l).

In linseed, when anthers were cultured on medium supplemented with BAP (1.0 mg/l) + IAA (2.0 mg/l) and the substitution of sucrose with combination sucrose-maltose significantly increased organogenesis in 'Mikael' and 'Barbara' but reduced shoots formation frequency of cultivar 'Dnepr-2' and completely inhibited shoots formation from anther derived callus of 'Lirina'. This indicates the high influence of genotypes on shoot regeneration. Better growth of calli was observed on the regeneration medium MS or B_5 with BAP. However, the influence of genotype on calli growth on this medium was not observed. It shows that for obtaining plants-regenerants important is not only composition of the regeneration medium, but even more crucial is the selection of used embryo induction medium (Mikelsone *et al*., 2011). The medium containing only 6% or 9% maltose had found to induce the highest overall efficiency of regeneration (Chen *et al*., 1998). Shoots formed on calli derived from the microspores inside the cultured anthers on media NN and N_6 supplemented with zeatin (1.0 mg/l) or BAP (1.0 mg/l) + NAA (1.0 mg/l), respectively and elongated on MS medium supplemented with 2 mg/l zeatin (Obert *et al*., 2004). Maximum shoot regeneration was achieved when the induced calli were transferred onto a modified N_6 medium containing zeatin (1.0 mg/l) (Nichterlein *et al*., 1991). Other than NAA and BAP, the modified MS medium containing thiamin hydrochloride (10 mg/1) had found to significantly increase the number of calli forming shoots/100 responded anthers and the overall efficiency of regeneration (Chen *et al*., 1998).

In Niger, shoot initiation was obtained from non-embryogenic callus on MS medium containing BAP (1.0 mg/l) and NAA (0.1 mg/l) (15-37%) (Sarvesh *et al*., 1993). These results are in line with the findings of Adda *et al*. (1994). Instead of using NAA with BAP, kinetin and BAP can also induce better shoot regeneration. Maurya *et al*. (2024) reported 19 per cent shoot initiation on MS medium containing BAP (0.5 mg/l) and kinetin (0.5 mg/l) followed by 3% shoot initiation on MS medium containing BAP (0.5 mg/l) and activated charcoal (0.5 mg/l). We can get direct regeneration of plantlets from embryogenic callus. For instance, matured embryos sub cultured to half strength B_5 medium supplied with 2,4-D (2.0 µM) + BAP (1.0 µM) with 2% sucrose found to germinate embryos into plantlets in three weeks (Murthy *et al*. 2000). For direct germination of embryos different sugars can also be used. Among the different sugars, sucrose had found to be the best one for conversion of mature embryos into plantlets on germination medium containing sucrose (0.09 M) (Hema and Murthy, 2007). Other than these hormonal combinations, Tesfaye *et al*. (2010) reported 83.3 per cent of shoot regeneration in MS medium supplied with IAA (1.0 mg/l) + kinetin (2.0 mg/l). These results are in line with the findings of Anusha (2024).

For *Brassica* species, Alam *et al*. (2009) reported that the medium supplied with MS + BAP (2.0 mg/l) + IAA (1 .0 mg/l) produced 56% of shoot regeneration. These results are in line with Sayem *et al*. (2010), who reported that the media combination MS + BAP (2.0 mg/l) + NAA (0.5 mg/l) showed the best performance for shoot regeneration. On that contrary, Nagoo *et al*. (2018) reported better shoot regeneration on MS full strength medium supplemented with kinetin (2.0 mg/l). On the other hand, the media, B_5 + BAP (4.0 ml/l) + NAA (1.0 ml/l) induced better shoot regeneration in Indian mustard (Reetisana *et al*., 2018).

Root formation

In anther culture studies, after shoot regeneration, the well-developed shoots are to be transferred to the rooting media for root regeneration. The commonly used growth regulators for root initiation are IBA, IAA and NAA. MS medium without any of these growth regulators can also induce roots based on the genotypes and the crop species.

Saji and Sujata (1998) observed rooting of shoots on half-strength MS medium supplemented with NAA (0.5 mg/l) in sunflower.

In safflower, rhizogenesis of the shoots has been observed on MS (half-strength) medium supplemented with NAA (0.1 mg/l) and 1% sucrose (Rajendra Prasad, 1991). Nakas *et al.* (2023) also carried out rooting in a half-strength MS medium involving NAA (0.1 mg/l) with 1% sucrose. However, Kassa *et al*. (2024) reported the largest mean root formation response (17.49 %) on MS medium containing IBA (1.0 mg/l). However, the cultured shoots had not form any roots in the medium lacking IBA (Anelay *et al*., 2024).

In linseed the most effective rooting (80%) was found on MS basal medium with IAA (2.0 mg/l) (Obert *et al*., 2004). Other than MS medium, the use of B_5 medium had also given positive results in initiation of roots. For instance, Nichterlein *et al*. (1991) rooted shoots on modified B_5 or MS media containing NAA (0.1 mg/l).

In Niger, many scholars have reported that MS medium without any growth regulators induces long and extensive roots (Adda *et al*., 1994 and Anusha, 2024). The same findings were also reported by Sarvesh *et al*. (1993) where a basal medium without growth regulators was found to be the best for further growth and rooting of the shoots. Maurya *et al.* (2024) reported that MS medium containing IBA (2.0 mg/1) was efficient for rooting with a frequency of 85 to 90 per cent in Niger but Anusha (2024) could only get 16.67 per cent of rooting in this media. In general, MS-rooting medium with IBA (0.5 mg/l) is the optimum concentration for rooting of shoots and it has also found to induce maximum average number of roots (Tesfaye *et al*., 2010).

For *Brassica* species, Alam *et al*. (2009) reported that among the three treatments, half MS medium supplemented with IBA (1.0 mg/l) + NAA (0.5 mg/l) was found to be the best (75.00%) for root initiation. Nagoo *et al*. (2018) reported maximum rooting (30.07%) when MS medium was supplemented with IBA (0.4 mg/l). On the contrary, Reetisana *et al.* (2018) reported the media composition MS + NAA (1.0 ml/l) + BAP (0.5 ml/l) showed highest per cent of root regeneration (45.83%) in Indian mustard.

Cytological analysis of the regenerated plants

Confirmation of ploidy level is most important in the final stage of haploid production programme. Ploidy level can be confirmed through either direct methods (chromosome counting) or indirect methods (flow cytometry, number of chloroplasts in guard cell, stomata size and morphological observations). Not only haploids or DHs have been obtained by *in vitro* anther culture. Non-haploid (diploid, triploid, tetraploid, pentaploid and hexaploid) embryos and plantlets have been obtained from anther culture of various genotypes (D'Amato, 1977 and Dunwell, 2010).

Cytological analysis of embryogenic callus and somatic embryos revealed haploids at a frequency of 30%, while that of rooted plants showed haploid regenerants at a frequency of 8.3%. (Saji and Sujata, 1998).

In safflower, the haploids were predominant (64%) followed by diploids (36%). The presence of other ploidy levels may be due to two events *viz.*, failure of wall formation in early microspore division and fusion of vegetative and generative nuclei (Chen, 1977). Prasad *et al*. (1990) done cytological studies of anther derived calluses and observed 47.3% to be haploid, 29.74% diploid and 16.36% triploid.

In linseed, cytological analysis of root tips of 13 randomly selected regenerated plants revealed the presence of four haploid and nine diploid plants (Nichterlein *et al*., 1991).

In Niger, Sarvesh *et al*. (1993) analyzed the chromosomes from the root tips of the regenerated plants obtained via embryogenesis and revealed the haploid nature in 85% of the plants. The remaining 15% included diploids (5%) and polyploids (10%). The same method was also followed by Adda *et al*. (1994) and reported that the plants were predominantly (127) haploids (n = 15), but eight plants turned out to be diploids. Similarly, the haploid nature of the plantlets was confirmed by chromosome analysis and 90% of the plantlets were haploids with n=15 chromosomes and remaining were diploids (Murthy *et al*., 2000) and twelve plants showed the haploid chromosome number (n=15) and the remaining found to be diploids. (Hema and Murthy, 2007). Maurya *et al*. (2024), used ploidy index using cell size and chromosome count, which revealed cell size of haploid cells 2 to 3 times less than diploid cells and chromosome number as $n = 15$ in haploid plants versus $2n = 30$ DH plants.

In *Brassica rapa*, Nagoo *et al*. (2018) conducted root tip mitosis chromosome counts and revealed maximum percentage of haploid frequency in KOS-1(41.85%) followed by KS-101(38.84%). Similarly, Reetisana *et al*. (2018) undertook cytological examination of the root tips of the plants regenerated from anther culture and found them to be haploids, that is n=16 (2n=36) in nature.

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