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Review

Induction of Haploidy from Pollen Grains in Angiosperms – the Current Status

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Summary. Since the successful induction of haploids from anthers cultured in vitro in 1964, a great deal of attention has been given to this problem by those interested in obtaining pure lines and mutants for crop improvement and biochemical genetics. In the last 16 years the anther culture technique has been refined and extended to over one hundred and fifty different species. More recently, isolated pollen culture – which is a refinement of the original anther culture technique – has also been developed. In this review we have made an effort to critically examine existing reports with the objective of analysing the effects of various factors – e.g. culture medium, the cultural conditions, and the effect of genotype and physiological state of the parent plant on pollen induction – and to speculate on the mechanism of action of different factors in order to throw some light on the process of haploid induction.

Key words: Haploids – Anther culture – Pollen culture – Physiological factors

Introduction

The phenomenal progress in the field of microbial genetics can be attributed to the haploid nature of microorganisms, whereas, in higher plants – which are diploid or polyploid – similar investigations have been greatly encumbered by the problems of dominance and segregation. Natural haploids of higher plants are of rare occurrence and restricted to only a few species (Kimber and Riley 1963). Geneticists and plant breeders have, therefore, long wished for dependable methods for the production of haploids. Haploids can be useful not only in obtaining mutants at a much higher frequency but one can also readily produce homozygous pure lines. Although in some special cases individual workers (e.g. Hougas and Peloquin 1957) obtained haploids experimentally by some special methods, until 1964 large-scale production of haploids in higher plants was only a theoretical possibility. However, with the publication of the paper by Guha and Maheshwari in that year, a new approach with general applications came into existence and, subsequently, in many laboratories a great deal of research effort was immediately directed towards perfecting the technique so that it could routinely be used. The plant which led to the initial success was a common weed, *Datura innoxia* Mill., belonging to the family Solanaceae. Anther cultures of this plant, developed from the pollen grains, resulted in embryoids which were haploid (Guha and Maheshwari 1966, 1967).

The investigations on *Datura innoxia* were soon extended to *Nicotiana tabacum* by Bourgin and Nitsch (1967), Nakata and Tanaka (1968) and others. The successful induction of haploid sporophytes in *Datura* and *Nicotiana* attracted the attention of scientists all over the world and, since then, numerous papers have appeared. To date, anther culture has been successful in about 153 species belonging to 52 genera and 23 families of dicots and monocots. Among the many subsequent investigators who have followed up this problem a few have attempted reviews of the earlier work, the main ones being those by Sunderland and Dunwell (1977), Nitzsche and Wenzel (1977) and Reinert and Bajaj (1977). However, the present review has been written with the viewpoint of examining the general physiology of pollen embryoid development so that we can determine factors that control androgenesis and extend success to other plants. Also, we have included results of newer investigations where androgenesis has been reported recently (Table 1).

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Table 1. Species in which anther culture has been reported after the year 1977. Response observed has been indicated by abbreviations: C for callus, PE for pollen divisions up to globular embryo formation and P for plantlet formation

Plant	Response	Reference
Aesculus hippocastanum	P	Radojević (1978)
Arabidopsis griffithiana	C	Amos and Scholl (1978)
A. korshinsky	C	Amos and Scholl (1978)
A. pumila	C	Amos and Scholl (1978)
Gerbera jamesonii	P	Preil et al. (1977)
Hevea brasiliensis	P	Chen et al. (1979a)
Hoscyamus muticus	P	Wernicke et al. (1979)
Luffa cylindrica	PE	Sinha et al. (1978)
L. echinata	PE	Sinha et al. (1978)
Nicotiana velutina	P	Vagera (1978)
Phaseolus vulgaris	C	Peters et al. (1977)
Physalis minima	P	George and Rao (1979)
Solanum melongena	P	Guy et al. (1979)
S. surattense	P	Sinha et al. (1979)
S. tuberosum (2x)	P	Foroughi-Wehr et al. (1977) Sopory et al. (1978)
Withania somnifera	C	Vishnoi et al. (1979)
Vitis vinifera x *V. rupestris*	P	Rajsekaran and Mullins (1979)

Culture Methods

Anther Culture

For the culture of whole anthers, flower buds are usually surface-sterilized and dissected to remove the anthers. During dissection, care should be taken to avoid injury to anthers as, in our experience, it stimulates callusing from the injured surface – the anther wall. The anthers can be cultured on the surface of solid medium (Guha and Maheshwari 1967) or floated on liquid medium (Wernicke and Kohlenbach 1975, 1976; Tyagi et al. 1979; Fig. 1). When dissection of buds is difficult, owing to their small size, an intact whorl of stamens, as in tobacco (Sunderland and Dunwell 1977), or a whole spike, as in barley (Wilson 1977), may be inoculated. The presence of other parts does not seem to affect the pollen response, at least in *Nicotiana tabacum*, if anthers are in direct contact with the medium (Sunderland and Dunwell 1977).

Pollen Culture

The technique of anther culture suffers from one main disadvantage – i.e. plants may originate not only from the

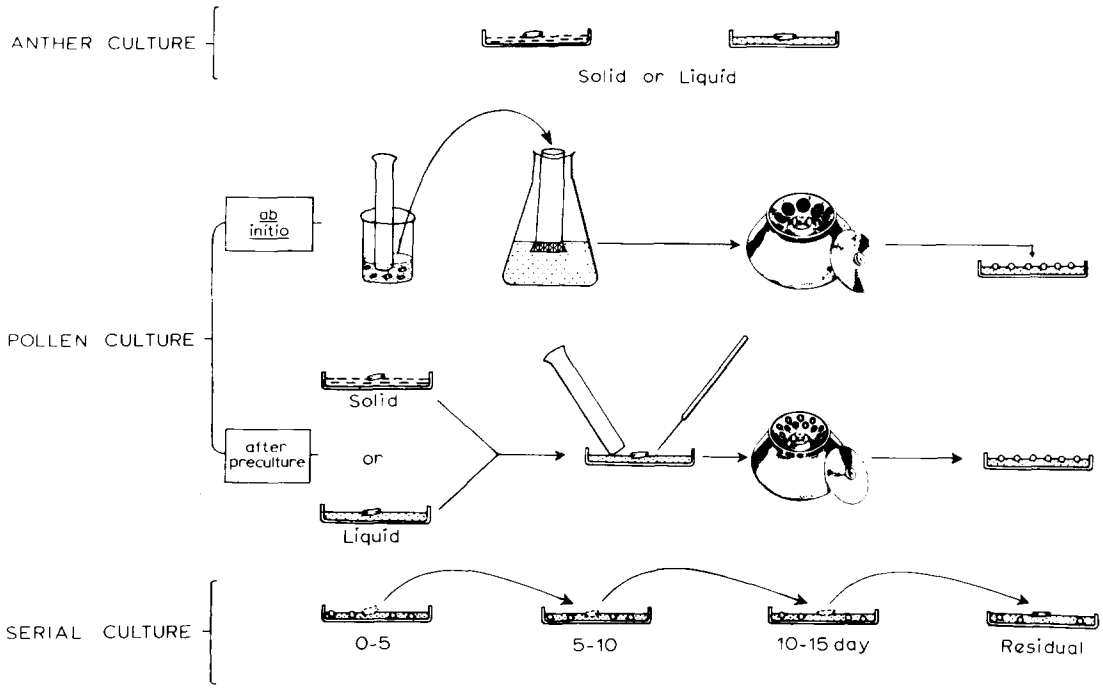

Fig. 1. Various techniques of induction of pollen embryos. Anthers are cultured on solid agar or liquid medium. *Ab initio* pollen culture: pollen are isolated by gently pressing the anthers with a pestle in liquid medium and filtering through sieve to remove coarse debris. They are subsequently washed with medium by centrifugation and plated. Anthers, precultured on liquid or solid agar medium, are dissected and pressed with pestle in liquid medium for pollen isolation. Pollen may be plated in the same medium or processed further as in *ab initio* pollen culture. Serial culture: anthers are transferred serially to new liquid medium. In each culture period anthers shed pollen into the medium; these continue their development into embryos

pollen grains, but also from various parts of the anther with the result that a mixed population of plants with various levels of ploidy may be obtained in a given culture. These plantlets of high ploidy originating from the diploid tissue cannot be utilized for further research. In addition to this, the anther wall may also have deleterious effects on the development of pollen grains into embryos (see Discussion). It is against this background that the culture of isolated pollen grains is being attempted by many workers. In addition to the certainty of the genetic purity of the plants, culture of pollen grains has potentially several other advantages, the main one being that they can be plated and manipulated like microorganisms and thus be very useful for studies on mutation and genetic transformation. However, it may be noted that culture of isolated pollen grains may not be so advantageous in some plants (e.g. certain lines of rye, Wenzel et al. 1976) which produce unreduced gametes. Here, some method for separating these unreduced heterozygous pollen (larger in size) may be applied before starting pollen culture as suggested by Wenzel (1980).

Although, divisions had been reported in cultured pollen grains by several workers (Niizeki and Grant 1971; Binding 1972; Sharp et al. 1972), fully developed embryoids were not obtained in any of these investigations. For this, credit is due to Nitsch and Norreel (1973) who first reported success in inducing embryoids from isolated pollen grains of *Datura innoxia* by adopting the technique shown in Figure 1 which we refer to as ab initio pollen culture. This technique has also been extended to *Petunia hybrida* (Sangwan and Norreel 1975). The frequency of responding pollen grains, however, is very low. Wenzel et al. (1975) and Sopory (1977) also made some attempts to culture the pollen grains of *Secale cereale* and *Solanum tuberosum*, respectively. However, only a few divisions could be obtained in both cases.

As *ab initio* pollen culture has worked only in a couple of species and here, too, the response has been low, workers are busy devising new methods for improving the frequency of embryoid formation. Nitsch (1974a), Reinert and coworkers (1975) and Wernicke and Kohlenbach (1977) modified the technique of Nitsch and Norreel (1973) for pollen culture of *Nicotiana tabacum* where pollen grains used for culture were isolated from precultured anthers (Fig. 1). Preculture of anthers was found essential for embryoid formation in this plant. In our experiments with *Datura innoxia* the frequency of pollen embryiod formation was only about 0.01% when pollen grains isolated from fresh anthers were cultured. However, it increased to 0.55% when pollen grains used for culture were isolated from 5-day old precultured anthers (Tyagi et al. 1979). It must be noted that in both cases anthers were taken from cold-treated buds. In rice also, multicellular calli have been obtained from pollen grains isolated from precultured anthers whereas no divisions could be obtained in ab initio pollen culture (Chen et al. 1979b).

More recently, Sunderland and Roberts (1977) have introduced another method of embryoid production from pollen grains. As developed for *Nicotiana tabacum*, this requires the culture of anthers in liquid medium where they dehisce and shed pollen into the medium. By transferring the anthers to fresh medium at intervals, a series of cultures of 'free' pollen is obtained which develop into embryoids (Fig. 1). When serial culture was started with anthers from cold-treated buds, embryoids were not only formed in all fractions of the shed pollen but the frequency was also considerably higher. This technique has also worked well with *Datura innoxia* (Tyagi et al. 1979) and a few divisions could also be induced in pollen liberated from anthers of rice (Chen et al. 1979b).

The experiments of Wenzel and coworkers (1975) offer further refinement in pollen culture technique in that they have succeeded in separating an enriched fraction of viable pollen grains of *Secale cereale* from the general population by layering the filtered suspension (macerated anthers passed through sieve, centrifuged and then suspended in the medium) over highly concentrated sucrose solution and centrifuging them. The active pollen made a layer over the sucrose solution while the non-viable ones settled at the bottom. Later, Wernicke and coworkers (1978) also tried similar experiments with *Nicotiana tabacum* pollen by subjecting them to density gradient centrifugation and employing 'Percoll' with sucrose. The separated fraction showed about a 40-fold enrichment in plantlet-forming microspores over the 'unfractionated' population. It is to be noted, however, that in both cases, this enriched fraction of pollen showed the embryogenic capacity only when pollen grains were taken from precultured anthers.

Factors Affecting Androgenesis

The main aim of anther culture is selective stimulation of pollen to divide and form either embryoids or callus. Various cultural conditions and even genetic factors, either singly or in an interacting manner, affect the pollen grains in their new developmental pathway. An identification of such factors is of utmost importance to obtain the desired results. The following pages contain a discussion of the different factors reported to affect embryoid formation in anther or pollen cultures and their possible modes of action.

Genotype of the Plant

Plant genotype is an important factor for the induction of androgenic haploids. Nitsch (1969) was able to obtain anther response in only 5 of the 12 species of *Nicotiana* tried. Polyploid species were more responsive than diploid

ones. Guha-Mukherjee (1973) reported that certain cultivars of rice were more responsive than others. Also, in *Arabidopsis thaliana* (Gresshoff and Doy 1972a) only 3 out of 18 lines, in *Lycopersicon esculentum* (Gresshoff and Doy 1972b) only 3 out of 43 lines, and in *Vitis vinifera* (Gresshoff and Doy 1974) only 3 out of 27 lines, responded to anther cultures. Similarly, of the 46 species of genus *Solanum* from which 118 clones and 9 interspecific hybrids were tested, only 19 species and 4 interspecific hybrids were able to produce pollen plants (Irikura 1975). The effect of genotype on anther response has also been reported by Wenzel et al. in rye (1977). In their experiments, anthers taken from hybrid plants of rye having a common pollinator, the Bulgarian short stalk mutant M II, yielded the highest numbers of pollen plants. Recently, Jacobsen and Sopory (1978), using a clone of *S. tuberosum*, made an attempt to accumulate the gene(s) favouring embryoid production. In their experiments they first cultured different clones and then, by intercrossing, succeeded in selecting a particular progeny strain which proved to be better in response to its parents. These experiments are further indicative of the influence of genotype on anther response.

However, some caution must be taken in ascribing failure in haploid induction in recalcitrant species to genotype, as more extensive work often proves rewarding. Thus, Amos and Scholl (1978) have been able to induce haploid callus in two *Arabidopsis thaliana* cultivars which Gresshoff and Doy (1972a) had earlier found unsuitable. Similarly, response has been observed in some species of *Nicotiana* (Tomes and Collins 1976) which were considered recalcitrant earlier by Nitsch (1969). In conclusion one can say that the genotype of the plant does have a strong effect on plantlet formation in anther cultures. However, it appears to be possible to avoid its deleterious effects, at least in part, by manipulating specific physiological conditions.

Physiological State of the Parent Plant

Success in haploid induction is in part dependent on a knowledge of the physiology of the pollen-yielding plant (see Sunderland and Dunwell 1977; Maheshwari et al. 1980). A significant correlation between plant age and anther response has been shown by various workers. The frequency of androgenesis is higher in anthers harvested at the beginning of the flowering period and declines with plant age in *Datura metel* (Narayanaswamy and Chandy 1971), *Nicotiana tabacum* (Sunderland 1971; Anagnostakis 1974; Dunwell 1976) and *Atropa belladonna* (Rashid and Street 1973). The reduced response has been ascribed to the deterioration in the general condition of the plants, particularly during seed set, as shown by experiments on *D. innoxia* in which the effect of plant age on anther response can be nullified when plants are prevented from seed-setting by removing the older flower buds (Nitsch 1975; Tyagi et al. unpublished work). The lower frequency of induction of haploids in anthers taken from older plants may also be associated with a decline in pollen viability (Sunderland 1971). The effect of plant age, however, needs to be looked into more closely since in *N. tabacum* L. cv. 'Havanna' no difference in androgenesis was observed between the first formed flower buds and the later developed ones (Johansson and Eriksson 1977).

Seasonal variations – in all likelihood working through temperature and light – have also been reported to affect anther response in *Solanum tuberosum* (Dunwell and Sunderland 1973) and *Triticum aestivum* (Picard and de Buyser 1975). In *Datura innoxia* (Nitsch 1975) and *Nicotiana knightiana* (Sunderland and Dunwell 1977) higher anther response was obtained when anthers were taken from plants grown at higher temperatures (20-30°C), whereas in *Brassica napus* cv. 'Tower' (Keller and Stringam 1978), the best results were obtained when anthers were taken from plants grown at lower temperature.

As for the effect of photoperiod received by the donor plant, a study on anther culture of *Nicotiana tabacum* cv. 'White Burley', revealed that if parent plants were grown under a 8 h regime at 14 Klux intensity, it resulted in 19.3 plantlets per anther and 56.6% anther response in comparison to 5.8 plantlets per anther and 34.9% anther response at a 16 h regime at the same light intensity (Dunwell 1976). Obviously, these interactions are not a simple matter of more photosynthesis or more growth but are more complex and probably photoperiodic in nature. Recently, Heberle-Bors and Reinert (1979) reported that in *N. tabacum* var. 'Badischer Burley', there was a five-fold increase in plantlet-forming pollen grains when the pollen grains were isolated from precultured anthers taken from plants growing under short-days rather than long-days. However, when instead of isolated pollen anthers were cultured as such, no difference could be observed, in contrast to the results of Dunwell.

In addition to seasonal variations, physical treatments and application of hormones and salts to the plant also alter its physiological status, which is reflected in a change in anther response. Of interest, in relation to the physical treatments, is the work on wheat by Picard (1973), who showed that removal of the apical region of the inflorescence increased the number of microspores having identical nuclei, which is correlated with enhanced embryoid formation on culture. More recently, it has been shown that in *Hordeum vulgare* a high frequency of similar divisions in pollen nuclei and callus formation could be achieved if plants were clipped at the ground level and allowed to stand in water for 1 or 2 days before culturing

the spike (Wilson 1977; Wilson et al. 1978).

Some evidence for a change in the physiology of donor plant by hormonal treatment can be seen in the experiments of Bennett and Hughes (1972) in which they sprayed 2-chloroethylphosphonic acid, an ethylene releasing compound, on wheat plants in the field, inducing abnormal nuclear divisions of both generative as well as vegetative nuclei in pollen grains which were in turn assumed to be conducive to embryoid formation. However, these pollen grains were not actually cultured and further experiments are needed to verify that the above assumptions are really correct. In *Oryza sativa*, application of 2-chloroethylphosphonic acid to inflorescences for 48 h at 10°C increased the anther response (Wang et al. 1974). Since, however, the sprayings were done at 10°C, the beneficial effects of cold treatment and 2-chloroethylphosphonic acid on androgenesis remain to be separated (see also Discussion). This is important because in several other plants 2-chloroethylphosphonic acid has been reported to cause abnormal nuclear divisions which bear no relationship to divisions in pollen grains (MacDonald and Grant 1974).

Strangely, Sunderland (1978) found that anthers taken from nitrogen-starved plants of *Nicotiana tabacum* cv. 'White Burley' gave better anther response as well as anther productivity when cultured than anthers taken from nitrogen-fed plants. However, Heberle-Bors and Reinert (1979), working with the another variety, 'Badischer Burley', of the same species found that a better nutritional supply of mineral salts increased anther response as well as the percentage of responding pollen grains in isolated pollen cultures.

Pollen Stage

The developmental stage of the pollen at which the anther is cultured is also a critical factor in embryogenesis. The maximum response in a majority of cases is obtained when pollen grains are cultured at the uninucleate stage or at the verge of mitosis. However, in pollen cultures of *Nicotiana tabacum*, some varietal differences have been recorded. The maximal plantlet formation occurred at the early binucleate stage in *N. tabacum* var. 'Badischer Burley' (Heberle and Reinert 1977; Heberle-Bors and Reinert 1979), whereas in *N. tabacum* cv. 'White Burley', pollen at the mitotic as well as the early binucleate stage were effective in producing embryoids with high efficiency (Sunderland and Roberts 1977, 1979). The stage of pollen is also important from quite a different viewpoint. It is now a well known fact that embryos and plantlets of higher ploidy are also produced along with haploids (Sunderland and Dunwell 1977). This in many plants is dependent on the stage of pollen at the time of inoculation. For example, in *Datura innoxia* (Engvild et al. 1972), *N. tabacum* (Engvild 1974) and *Hyoscyamus niger* (Corduan 1975), it has been shown that plantlets obtained from pollen at the uninucleate stage were mostly haploids, whereas, at later stages, higher chromosome numbers were found. In general, the older the stage selected, the higher the ploidy level of the resulting embryoids. In *D. innoxia*, which is a highly responsive species, 80% of the embryoids obtained from binucleate microspores were non-haploid (Sunderland et al. 1974). In cereals, usually an uninucleate stage has been preferred in anther cultures, but even then plantlets of higher ploidy are commonly obtained. This may be due to aberrations in mitosis during the callusing phase or due to nuclear fusion during early division of pollen grains as shown in barley (Sunderland et al. 1979).

Pretreatment to Flower Buds

Certain physical and chemical treatments given to flower buds or anthers, prior to culture, can be highly conducive to the development of pollen into plants. The most significant is cold treatment. Such treatment given to flower buds before inoculation enhanced anther response – indeed, it was found obligatory for successful pollen culture in *Datura innoxia* (Nitsch and Norreel 1973). This enhancing effect of cold treatment has already been reported in several plants. The work in our laboratory on *Petunia hybrida* (Malhotra and Maheshwari 1977) and *D. innoxia* (Tyagi et al. 1979) also supports this observation. Some workers have given cold treatment to isolated anthers after culturing them (Duncan and Heberle 1976; Sunderland and Wildon 1979). Although enhancement in anther response has been invariably recorded, cold treatment is generally more effective when given to flower buds (Nitsch 1977; Sunderland and Wildon 1979).

In most of the earlier reports the dose of cold treatment has been selected arbitrarily. A cold treatment of 48 h at 3°C has been recommended for *Datura innoxia* by Nitsch (1974b). However, in our experiments, where a detailed study was carried out, cold treatment at 4°C for 4 days has been found to be the optimal one (Tyagi et al. 1979; details under publication). For *Nicotiana tabacum* flower buds, Nitsch (1974a) has recommended cold treatment for 72 h at 5°C, but Sunderland and Roberts (1977) found a 12-day cold treatment at 7-8°C to be optimal. In *Hyoscyamus niger*, pretreatment at 15°C for 5 days has been found to be better than other treatments tried (Sunderland and Wildon 1979). In most cases, such as *D. innoxia* (Nitsch 1977; Tyagi et al. 1979) and *H. niger* (Sunderland and Wildon 1979), the same cytological stage of the pollen – prior to mitosis – has been reported to be the best responding one whether the anthers were

cultured before or after pretreatment. However, in *H. albus* the best responding stage for the culture of untreated anthers is the miduninucleate stage, and for pretreated anthers, it is the early binucleate stage. This shift in the optimal response after cold treatment is peculiar and without any satisfactory explanation although it is thought that this might be due to varying contents of inhibitors during pollen development and which may be differentially affected by cold treatment at different stages (Sunderland and Wildon 1979).

How cold treatment enhances the frequency of embryoid formation is an interesting question but, unfortunately, at this moment one can do little more than speculate. According to Nitsch (1974b) cold treatment increases the frequency of embryoid formation by increasing the number of pollen with similar nuclei, and maintains pollen in viable condition. In her experiments on *Datura innoxia* after 5 days of culture 22% of the microspores of the cold-treated anthers showed identical nuclei in comparison to only 3% in non-treated anthers. In addition to this, the number of the dead grains in the cold-treated anthers was 62% in comparison to controls where it was 92%, while no dead pollen grains were observed in fresh or cold-treated anthers before culture.

Duncan and Heberle (1976) who gave cold treatment to the anthers after inoculation instead of prior to inoculation, also observed an increase in viable units after cold treatment but did not find any evidence to support the idea that cold treatment causes symmetrical division in a pollen grain.

Cold treatment also delays senescence of anthers (Pelletier and Henry 1974) and rapid senescence of somatic tissue is known to be inhibitory to the production of haploids (Pelletier and Ilami 1972; Mii 1976). So, it could also be one of the explanations for increase in embryoid frequency by cold treatment.

It must be confessed that the actual biochemistry of the effect of chilling still remains to be worked out. An article of great relevance is one which reports that low temperature leads to dissolution of microtubules (Hepler and Palevitz 1974). Since the latter are involved in spindle formation, it is conceivable that cold treatment may exert its effect through an early interference in establishment of polarity, thus resulting in a change in spindle organization. Recently Sangwan-Norreel (1977) tried to replace the low temperature effect by centrifuging the buds of *Datura innoxia*; this procedure did improve the anther response to some extent (increase of 6%). The effect is accentuated when cold treatment is combined with centrifugation (increase of 31%). It is of interest that centrifugation is also known to cause disorganization in the assembly of microtubules (Hepler and Palevitz 1974). However, in what way the breakdown of microtubules – if it really occurs in pollen grains – is beneficial for embryoid induction still remains to be understood. Sangwan and Camefort (1978) have observed that in *Datura metel* and *Nicotiana tabacum* cold treatment brings about an increase in total free amino acids and a decrease in bound amino acids.

Culture Medium

Various mineral media, like those of Murashige and Skoog (1962), White (1963), Linsmaier and Skoog (1965), Blaydes (1966) and Nitsch (1969), have been used for different species. It is hard, however, to draw general conclusions concerning which medium from the above-mentioned is the most suitable and the serious investigator is advised to consult the detailed monograph of Nitzsche and Wenzel (1977) where all the relevant information on this point has been compiled.

Until the early seventies, the agar solidified medium was routinely employed for anther culture. Recently, however, Wernicke and Kohlenbach, have reported that anthers of *Scopolia carniolica* (1975) and *Nicotiana tabacum* (1976) give better response in liquid medium. Similar results have also been obtained in *Datura innoxia* (Tyagi et al. 1979). The enhanced response in liquid medium may be attributed to any one or a combination of postulated factors viz. removal or better diffusion of inhibitory factor(s) present in anther wall, better supply of nutrients, or elimination of substances inhibitory for androgenesis that agar may itself contain (Wernicke and Kohlenbach 1976). The last point is further discussed in the following paragraphs.

The addition of activated charcoal to agar-solidified medium has been found to greatly enhance the response in anther culture of *Nicotiana* species (Nakamura and Itagaki 1973; Anagnostakis 1974; Wernicke and Kohlenbach 1976), *Anemone virginiana* (Johansson and Eriksson 1977), *Secale cereale* (Wenzel et al. 1977), *Solanum tuberosum* (Sopory et al. 1978) and *Datura innoxia* (Tyagi et al. 1980a). Charcoal possibly affects the response by adsorbing inhibitory components present originally in the agar or released from the senescing anther wall. The possibility of inhibitors being present in the agar is based on the fact that a higher anther response was obtained when either the highly purified gelling agent 'agarose' or agar after dialysis against activated charcoal was used (Kohlenbach and Wernicke 1978). For the anther culture of *D. innoxia*, solid medium containing charcoal has also been found to be better than liquid medium (Tyagi et al. 1980a). For some reason, not yet clear, charcoal supplied in liquid medium inhibits the anther response, as noted in *N. tabacum* by Wernicke and Kohlenbach (1976) and in *D. innoxia* by the authors (Tyagi et al. 1980a). In any event, the positive results in solidified media suggest that charcoal not only removes inhibitory factors present in agar medium but possibly removes also the inhibitory factors elaborated by anthers.

Although it is certain that inclusion of charcoal in the medium enhances anther response, the precise mechanism of its effect on androgenesis is not yet clear and needs further work. Recently, Weatherhead et al. (1978) have

shown in *Nicotiana tabacum* that addition of charcoal to the medium enhances the anther response possibly by overcoming the deleterious effects of 5-hydroxymethylfurfural (HMF), which is produced by the degradation of sucrose during autoclaving of the medium. However, there may be other inhibitors that have not yet been identified. While studying properties of activated charcoal the same workers have found that it adsorbs auxins as well as cytokinins and they think that inclusion of hormones to charcoal containing medium may not be of much use. However, in contrast to this, Sopory (1979) obtained a better anther response in *Solanum tuberosum* on a medium containing both hormones and charcoal. In our view these experiments require further attention in order to reach a general conclusion.

The addition of glutamine (Nitsch and Norreel 1973; Keller et al. 1975; Wernicke and Kohlenbach 1977; Sunderland and Roberts 1977; Tyagi et al. unpublished) and glutathione (Nitsch 1969; Wenzel et al. 1977; Tyagi et al. unpublished) to the culture medium also enhances the embryogenic response. In addition to these, various natural though undefined extracts, e.g. coconut milk (Guha and Maheshwari 1964, 1966), potato extract (Anonymous Chinese Workers 1976; Wenzel et al. 1977; Sopory et al. 1978) and extract of anthers themselves (Nitsch and Norreel 1973) have also been used. The mode of action of these complex mixtures is not clear, but, they probably provide some substance(s) beneficial for cell divisions in pollen grains.

Sucrose

In most of the species investigated the best results were obtained with sucrose at a 2-3% concentration. However, concentrations up to 6-12% have also been used in anther cultures of *Hordeum vulgare* (Clapham 1971, 1973), *Triticum aestivum* (Ouyang et al. 1973; Wang et al. 1973), *Brassica campestris* (Keller et al. 1975) and *Solanum tuberosum* (Sopory et al. 1978). In *T. aestivum*, 6% sucrose enhances callus formation from pollen but inhibits it from somatic cells (Ouyang et al. 1973). At higher concentrations, sucrose – in addition to being a carbohydrate source – may possibly play some other role (osmoregulatory?) at the time of induction. However, once growth has begun, the high level has no further beneficial effect and is, in fact, deleterious. Transfer of anthers from a high to a low level of sucrose has been recommended in anther cultures of *B. campestris* (Keller et al. 1975) and *S. tuberosum* (Sopory 1979) for optimal post-induction growth. In the pollen culture of *Petunia hybrida*, where only a few divisions were obtained, an 0.25M concentration of mannitol with 0.05M sucrose has been found to be most effective (Binding 1972). In *S. tuberosum*, sucrose has been used together with mannitol for pollen culture (Sopory 1977).

Hormones

In a few species, namely *Datura innoxia* (Sopory 1972), *Nicotiana tabacum* (Nitsch 1969), *Hyoscyamus niger* (Corduan 1975; Raghavan 1975) and *Saintpaulia ionantha* (Hughes et al. 1975), embryoids develop even in the basal medium. In the vast majority of reports, however, either one or another hormone has been found necessary for an androgenic response. Even for the few plants named above, the addition of hormones has been found to be definitely beneficial. Auxins, cytokinins as well as gibberellins have been found to be stimulatory in these species (Nitsch 1969; Sopory and Maheshwari 1976b). Unfortunately, however, studies are not sufficiently detailed in most plants to enable a judgement of the relative efficacy of these hormones. Cytokinins have been found to be more efficaceous in *D. innoxia* (Sopory and Maheshwari 1976b) and *Solanum tuberosum* (Sopory et al. 1978), whereas in *N. tabacum* (Nitsch 1969) and *Lycopersicon esculentum* (Debergh and Nitsch 1973) auxins seem to be more effective. Similarly, the auxin 2,4-D has been found to promote anther response in *H. niger* by specifically inducing callus growth in certain pollen grains which otherwise would remain non-embryogenic (Raghavan 1978). In some plants, particularly cereals, the simultaneous presence of both an auxin and a cytokinin appears to be necessary (see Clapham 1977). This seems to be correlated with the somewhat unusual mode of embryoid development in cereals where the pollen grains initially produce calluses which can only later be differentiated into embryoids. For the first step auxin is required but for the second, only the basal medium (Niizeki and Oono 1971; Clapham 1973) or a cytokinin in combination with the auxin (Ouyang et al. 1973; Anonymous Chinese Workers 1974) is necessary. However, this mode of development greatly enhances the probability of chromosomal aberrations. Therefore, one should always look for those combinations of hormones which can increase the chances of direct development of the embryoids.

Nitsch (1974b) has reported that in pollen cultures of *Datura innoxia* hormones seem to be unnecessary for the induction of divisions in pollen: in the basal medium approximately 8% of the microspores yielded plantlets. With the addition of the hormones to the medium, response was, in fact, inhibited: with indoleacetic acid only about 0.8% and with zeatin only 0.08% of the microspores produced plantlets. This observation is in sharp contrast to those made on anther cultures in *D. innoxia* where the best response was observed using a medium containing zeatin (Sopory and Maheshwari 1976b). The finding of Nitsch (1974b) needs to be extended, since in the pollen culture of *Petunia hybrida*, response was obtained when NAA and benzyladenine were added to the medium (Sangwan and Norreel 1975).

Physical Factors

In the early studies, not much attention was paid to the possible role of physical factors but now they are considered to be important for the process of induction of haploids. Consider light first: it does not appear to be necessary for the induction process per se but its influence on post-induction growth has been reported in many plants. An alternating light and dark regime is beneficial for increased embryoid formation in *Hyoscyamus niger* (Corduan 1975), *Datura innoxia* (Sopory and Maheshwari 1976a) and *Nicotiana tabacum* (Sunderland 1971). For *D. metel*, continuous light was used (Narayanaswamy and Chandy 1971) whereas in *Anemone virginiana* continuous light inhibited embryoid formation (Johansson and Eriksson 1977). In *Vitis vinifera*, although, induction of callus was possible even in darkness, the frequency of callus induction was markedly enhanced if anther cultures were kept in continuous light for the first 24 h before transferring them to dark (Gresshoff and Doy 1974). For pollen culture of *D. innoxia* (Sangwan-Norreel 1977) and *N. tabacum* (Sunderland and Roberts 1977), an initial incubation in dark followed by diffuse light was found to be suitable.

Although the above examples make it obvious that light has a definite effect in anther culture and pollen culture, its precise regulatory role is far from clear in view of the obvious contradictions, i.e. of stimulatory action in certain plants and inhibitory in others. There are a couple of reports where workers – by illuminating with light of different wave-lengths – have tried to find out the regulatory role of light but they are not detailed enough to draw any major conclusion. For pollen culture of *Nicotiana tabacum* red light was more suitable than blue, low intensity white light or darkness (Nitsch 1977). On the contrary, in anther cultures of *Datura innoxia*, no embryoids were formed in red light (Sopory and Maheshwari 1976a).

The optimal temperature for the induction of embryoids lies between 25-30°C in *Nicotiana tabacum* (Sunderland 1971) and in *Datura innoxia* (Sopory and Maheshwari 1976a), and at lower temperatures, the anther response decreases markedly. However, in rice even though callus induction is favoured by high temperature (30°C), the induced callus showed reduced differentiation capacity (Anonymous Chinese Workers 1974). Likewise, in *Brassica napus* (Keller and Armstrong 1978), a higher temperature, 30°C in the first 14 days of culture, and in *B. campestris* (Keller and Armstrong 1979), 35°C for one to three days, followed by 25°C for rest of the culture period results in a higher yield of microspore derived plants consisting mainly of haploids. It is interesting to note that in their earlier work on the same plants (Keller et al. 1975; Keller and Armstrong 1977) no haploids were formed when anthers were cultured at 25°C from the beginning of culture. In addition to light and temperature many other factors, such as the manner of positioning of anthers on solid medium (Sopory and Maheshwari 1976a), number of anthers inoculated per culture vessel (Fouletier 1974; Michellon et al. 1974) and culture vessel atmosphere (Dunwell 1979) may also affect androgenic response.

Role of Anther Wall

As induction of the pollen into embryoids occurs most easily within the confines of an anther, it is important to understand the role of the anther wall. There are two schools of thought regarding the role of the anther wall. Nitsch and Norreel (1973) found the boiled water extract of cultured anthers of *Datura innoxia* to be effective in promoting growth of isolated pollen, indicating thereby that the anther wall plays an important role. Further, the stimulatory effect of the extract has been assigned to amino acids. Glutamine and serine, added to the culture medium, have been shown to particularly enhance the frequency of pollen embryoid formation (Nitsch 1974a). An analysis of the amino acid components of anthers of *Nicotiana tabacum* has shown that the level of glutamine increases by 4-5 fold during culture relative to that found in fresh anthers (Horner and Pratt 1979), although no significant changes in the levels of serine have been observed. Incidentally, the high frequency of embryoid formation from isolated pollen grains – when they are taken from the precultured anthers (page 3) – also suggests that the anther wall does have some stimulatory effect.

At variance with the stimulatory role, some workers speculate that some inhibitory factors emanate from anthers at the time of culture (Heberle and Reinert 1977; Tyagi et al. 1979). In studies on *Datura innoxia*, serial transfer of anthers to fresh medium enhanced the frequency of embryoid formation from pollen grains liberated in different fractions, two to three times, in comparison to continuous cultures where the anthers were kept in the same medium throughout the culture period (Tyagi et al. 1979). It can therefore be suggested that transfer of anthers to fresh medium dilutes out the inhibitor(s) and thus allows successive fractions of pollen grains to develop more readily into embryos.

Senescence of anther, as indicated by its browning, also inhibits plantlet formation (Pelletier and Ilami 1972). In addition to this, it has been found that senescing anthers not only fail to produce embryoids themselves but also retard the growth of potentially embryogenic pollen grains liberated from responding anthers in serial cultures of *Datura innoxia* (Tyagi et al. 1980b).

It may be that the anther wall has both beneficial as well as inhibitory effects. In the initial period of culture

it might be producing some factors which induce the androgenetic pathway of pollen development since pollen isolated from precultured anthers give better embryogenic response than *ab initio* cultured pollen. On the other hand, during the later period of incubation, when the anther wall starts to senesce even in the responding anthers, it might be releasing inhibitors which do not allow further growth of induced pollen grains. The identification of stimulatory or inhibitory factor(s), however, needs much intensive work. It would then be easy to induce haploids in species which have been recalcitrant so far either owing to suboptimal or supraoptimal levels of these factors.

Concluding Remarks on Factors Affecting Androgenesis

The information available on the optimal conditions for embryoid induction seems to be often contradictory and complicated as most workers have used conditions according to the facilities available to them and very few attempts have been made to draw general conclusions from such studies. However, from the discussion on the preceding pages, some generalizations can be extracted which may serve as guidelines for new workers in this field. To start with, anthers must be taken from a healthy plant growing in a suitable environment. A chilling pretreatment to flower buds or anthers often enhances success in embryoid induction. During culture, the degree of success depends greatly on the choice of a suitable liquid or agar-gelled medium and of various adjuvants to it, especially hormones. Addition of charcoal is often beneficial in agar-gelled media. A higher concentration of sucrose may also help in the induction process although for further development a lower sucrose concentration is desirable.

Mode of Androgenesis and Determination of Embryogenic Potential of Pollen Grains

Many workers, especially cytologists, have been interested in working out the exact mode of androgenesis. The interested reader is recommended to study reviews from Sunderland's laboratory (Sunderland and Dunwell 1977) and we shall not refer to this matter here. However, the real information about the changes taking place during the early phases of androgenesis at the subcellular level is extremely limited and the precise mechanism of induction of embryos in anther culture is still a mystery. Answers to questions in this regard have been particularly difficult to find because androgenesis is a highly variable response and even in the most favourable circumstances not all the pollen grains present in an anther respond to produce embryos or callus masses. Moreover, a high proportion of microspores soon die in anthers upon culture. One possible way to develop a controlled system is to obtain a highly purified fraction of viable pollen (as mentioned on page 3) and determine the conditions favouring their development into embryos.

In cultured anthers of *Nicotiana tabacum*, pollen grains can be identified into two types based on their size and staining properties: Type 1 – Pollen grains of large size which are densely stained with acetocarmine, and Type 2 – Pollen grains of small size which are lightly stained with acetocarmine. It is the Type 2 pollen grains from which embryoids originate (Sunderland and Wicks 1971). This observation has been further supported by Wernicke and coworkers (1978) in *N. tabacum* var. 'Atropurpurea'. However, Horner and Street (1978) have stated that dimorphism in pollen grains of *N. tabacum* exists even before culture and the culture process simply provides an environment which accelerates the expression of the predetermined potential for sporophyte development. A similar kind of dimorphism, before culture, has also been reported earlier in *Paeonia hybrida* and in the Sabarlis (Sunderland 1974) and Akka (Dale 1975) cultivars of *Hordeum vulgare*.

However, with regard to size and staining properties no distinction is possible between embryogenic and non-embryogenic pollen in *Datura innoxia*, either before culture or during culture of anthers, and one wonders if the argument for dimorphism being responsible for determination of the embryogenic or non-embryogenic capacity of a pollen grain is really valid. Sunderland (1974) tried to find some other differences in *D. innoxia* and has suggested that microspores arising from tetrads – having an arrangement with the two pairs of spores at right angles to each other – follow that route of androgenesis in which the first pollen mitosis results in two similar cells and both these cells equally participate in the embryoid formation. On the other hand, tetrads with simple tetrahedral arrangement give rise to pollen which follow a route in which pollen mitosis results into vegetative and generative cells and the vegetative cell alone forms the complete embryoid. It should be mentioned that in *D. innoxia*, androgenesis frequently follows the first route. It is not yet known how the configuration of the tetrads can determine the embryogenic potential of pollen.

An Assessment of the Use of Haploids

The most extensive trials for use of haploids for agricultural purposes appear to have been made by scientists in Japan and China. In Japan, an excellent commercial variety of tobacco (*Nicotiana tabacum*), 'F211', has been produced by anther culture. This variety is more resistant to bacterial wilt and has a mild smoking quality in comparison to the existing leading varieties of tobacco (Nakamura et al. 1974). Various research organisations in China have also produced high yielding and superior varieties of tobacco – 'Tanyu 1', 'Tanyu 2' and 'Tanyu 3'. Extensive work has also been done on rice where three new varieties

have been produced, namely – 'Huayu 1', 'Huayu 2', 'Tanfong 1', and in wheat where 'Haupei 1' and 'Lunghua 1' have been produced (see Hu et al. 1978). In *Brassica napus* also, anther-derived doubled haploid lines are now available and are under field test in Canada (Keller and Stringam 1978). Considerable work has been initiated upon in recent years in West Germany on haploid breeding of rape, rye and potato (Wenzel 1980). Wenzel et al. (1979) have proposed a detailed and interesting scheme of breeding in *Solanum tuberosum*, utilizing anther-derived haploids and somatic cell hybridization, in addition to the conventional methods of plant breeding. The scheme shows how autotetraploid potato could be reduced to the monohaploid level by anther culture and how then, by controlled combinations, a new synthetic heterozygous tetraploid potato with desirable traits might be produced.

As for selection of mutants and work directed towards biochemical genetics, Carlson (1970) for the first time made use of haploid callus for raising auxotrophic mutants of *Nicotiana tabacum* which required hypoxanthine, biotin, p-aminobenzoic acid, arginine, lysine or proline for their normal growth. Unfortunately, however, all of these turned out to be leaky. The author regards his failure either to be due to inefficiency of the technique for selecting true mutants or the allotetraploid nature of *N. tabacum*. In 1973, Carlson isolated methionine-sulfoximine resistant lines in *N. tabacum* which were resistant also to the wild fire disease caused by *Pseudomonas tabaci*. Following Carlson's work, several other workers have also isolated and characterized mutants resistant to various metabolic inhibitors, environmental stresses, herbicides and phytopathotoxins, utilizing pollen-derived haploid cell cultures (see Maliga 1978).

More recently, Müller and his coworkers in East Germany have initiated a study of the regulation of nitrate reductase in higher plants by making use of mutants derived from haploid cell cultures of *Nicotiana tabacum*, which contained more than 50% haploid cells even after four years of culture (Müller and Grafe 1978). This represents the most intensive study so far undertaken to examine, by means of anther derived haploids, the mode of regulation of genetic activity at the molecular level in higher plants. They isolated chlorate-resistant cell lines after mutagenic treatment of cells with N-ethyl-N-nitrosourea. These mutants lacked a normal nitrate reductase enzyme as judged by their inability to grow on nitrate when it was provided as the sole source of nitrogen in the culture medium. However, it was found to be possible to restore nitrate reductase activity in vitro from the selected nitrate reductase mutants by mixing extracts of nonallelic mutants (Mendel and Müller 1978). Recently, complementation has also been demonstrated by fusing protoplasts of two different mutants (Glimelius et al. 1978) and selecting hybrids on a medium containing nitrate as the sole source of nitrogen.

Problems and Prospects

It should be evident from the preceding paragraphs that there has been intense research activity in the general area of production and utilization of haploids. However, the overall impression one gains is that real progress is still very limited. There are several reasons for this, the most important being that the anther culture technique works well on plants belonging to only a few families such as Solanaceae. Even where response has been obtained the frequency of formation of embryoids is often very low. A second problem is that one often encounters plantlets of high ploidy levels whose precise mode of origin is uncertain and which cannot be utilized for further research (see Sunderland and Dunwell 1977). And, even when haploids are obtained, the continued maintenance of the haploidy is often a serious problem. Although para-fluorophenylalanine has been suggested to preferentially support the growth of haploid cells by selective inhibition of growth of cells of higher ploidy (Gupta and Carlson 1972), this property needs to be checked critically because of considerable disagreement in the results of various workers (Dix and Street 1974; Matthews and Vasil 1975). Further, in many species – especially the cereals – the problem is that the initial response of pollen to culture is of callus formation from which, only later, can plantlets be differentiated. In this embryogenic mode of development, the probability of chromosomal aberrations is very high. Moreover, in cereals, most of the plants obtained in anther culture turn out to be albinos (Nitzsche and Wenzel 1977).

In our view, the solution of the above mentioned problems lies in the use of isolated pollen culture. In this regard the serial culture technique is quite promising, but the utility of haploids can best be realized by the perfection of the technique of ab initio pollen culture. Success in this direction appears to be just a matter of more time and effort. With this technique it will be possible to plate the pollen, treat them like microorganisms, and obtain calluses or plantlets from them at will. However, in order to achieve this aim a great deal of basic research – perhaps extending over a period of another decade – appears to be necessary. The most basic of all problems is the proper understanding of the process of induction of haploids, i.e. how pollen grains are triggered into a phenotypically sporophytic mode of development instead of a gametophytic one. This study will not only provide knowledge for obtaining higher induction frequencies but also for facilitating the induction of haploids in non-responding species.

A way has already been shown by Wenzel et al. (1975)

by their work on *Secale cereale* and by Wernicke et al. (1978) on *Nicotiana tabacum* var. 'Atropurpurea'. Such a technique allows for the separation of a pollen fraction which has a high potentiality for formation of haploids. Isolation of such a fraction may in itself facilitate induction of haploids in recalcitrant species where inhibitors may be leaching from dying pollen grains and affecting adversely the growth of competent microspores. Moreover, with this technique one can now proceed to analyse the physiological and biochemical differences between the responding and non-responding pollen fraction, and this would give some insight into the process of induction of haploids.

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