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Review

In vitro haploid formation from pollen: a critical review

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Summary. In the field of regeneration of plant in vitro cultures, haploid formation from pollen is the scientifically most advanced, but at the same time very controversial system. In the present state of transition from basic research to commercial application, a sound scientific basis of pollen embryogenesis would make this transition much easier. New discoveries in recent years have made it possible to develop a new view of pollen embryogenesis. The new view includes recognition-theoretical aspects, provides a model for a number of problems in plant development, and has consequences for strategies for haploid production. The accumulated knowledge in the field of pollen plant formation is critically analyzed against this new view.

Key words: Anther culture – Embryogenesis – Haploids – Pollen – Pollen culture

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Historical

In the early days of plant tissue culture, any part of the plant was put into culture including the male sex organs, the anthers. However, as was the case with other cultured organs, the result was not what was expected. Guha and Maheshwari (1964, 1966) wanted to study normal male sexual development, but what developed in their cultured anthers were not male sex cells (pollen) but embryos (Maheshwari et al. 1980, 1982). These embryos developed into plants with a haploid (gametic) chromosome number.

Haploids had been known of for a long time before this discovery (Blakeslee et al. 1922) as had their potential uses in plant breeding and basic research (Blakeslee and Belling 1924). However, haploids from pollen opened a new dimension because of the abundance of pollen produced by the plants and their potentially general occurrence in the plant kingdom, thus overcoming the limitations of other sources of haploids (Nitzsche and Wenzel 1977).

The next 15 years saw people trying to trigger embryogenesis in pollen of many economically and scientifically interesting species. They did this largely in the tradition of tissue culture by manipulating the culture conditions of anthers or isolated pollen. Some Solanaceous species responded very well. In most other species, however, yields were disappointingly low or they were totally recalcitrant. A number of factors could be identified which enhanced pollen plant production in anther cultures (Maheshwari et al. 1980, 1982), but as compared to the potential of available pollen, the increases were meager. Others tried to liberate the pollen from the constraints of the surrounding anther wall in order to have better manipulatory access to the pollen (Nitsch and Norreel 1973; Nitsch 1974a; Reinert et al. 1975), but again the increases were not dramatic.

A similar situation existed at the same time with plant regeneration from cereal leaves. There, the collective knowledge of plant tissue culture was not able to trigger tissues or protoplasts from mature leaves into regeneration (Potrykus et al. 1976; Potrykus 1980). The break-through came with the recognition that regeneration can be achieved only from immature (Wernicke and Brettell 1980) or embryonal tissue (Vasil and Vasil 1981).

P-grains and the donor plants

With pollen embryogenesis, things were more complicated. There, the pollen to be cultured was immature from the beginning of experimentation, and paradoxically, one had to look into mature anthers in order to find a break-through. It was found that pollen grains which had similar staining and size characteristics as embryogenic pollen grains (P-grains) in cultured anthers (Sunderland 1971) also existed in mature anthers in situ (Wenzel and Thomas 1974; Sunderland 1974; Dale 1975; Horner and Street 1978). This phenomenon has been called pollen dimorphism. A thorough investigation in tobacco then led to the conclusion that in vitro conditions do not increase the frequency of P-grains, but that their frequency is predetermined before culture (Horner and Mott 1979) by the growth conditions of the donor plants (Heberle-Bors and Reinert 1979, 1981). Separation of the P-grains from the normal pollen grains isolated from optimally treated plants gave final proof for the identity of P-grains found in vitro and in vivo, and introduced direct pollen culture as a new culture procedure (Heberle-Bors and Reinert 1980).

To relate regeneration to processes taking place before explant excision is common place in conventional vegetative propagation, but it is a new approach for in vitro regeneration and raises both fundamental and application-oriented questions. What makes the pollen grains different? When and how does the acquisition of morphogenic competence occur? Which factors trigger this acquisition? Inevitably, a further question arises of why P-grains are formed. Or, in a teleonomic sense, what is the purpose of the P-grains for the donor plants since P-grains can develop into embryos only in vitro but not in vivo?

It turned out that P-grains in tobacco were functionally sterile pollen grains for the donor plants and that their frequency was increased by short days and low temperature. The same conditions also shifted sex balance in the flowers towards femaleness and reinforced floral induction of the donor plants (Heberle-Bors 1982a). Recently, the correlation between pollen embryogenesis and floral induction has been confirmed for *Brassica napus* (Thurling and Chay 1984). These correlations could be fitted into a rule which exists for many plants. Conditions of strong floral induction shift sex balance towards femaleness and increase pollen sterility (Heslop-Harrison 1957, 1972). This rule allowed predictions for the induction of P-grains in other species. Feminization and male sterility can depend upon a number of conditions, comprising periphysis, cyclophysis and topophysis of the plants, and a lot of knowledge has accumulated on this matter (see Heslop-Harrison 1972; Frankel and Galun 1977). For instance in cucurbits or many trees, there exist spatial or temporal patterns of sex balance. There, one would suggest taking only those flowers for anther or pollen culture which are moderately feminized so that male development is not completely suppressed and the formation of sterile and possibly embryogenic pollen occurs.

Further studies revealed that P-grain induction did not take place during floral induction, but early in flower development, at the same time as the shift in sex balance towards femaleness – during the meiotic prophase (Heberle-Bors 1982b). The identification of the inductive stage then allowed the induction of P-grains by chemical treatments of tobacco plants with agents known to feminize flowers in other species (Heberle-Bors 1983). Such feminizing factors were auxins, antigibberellins (Alar), and ionic effects such as nitrogen starvation. Recently, the Alar effect has been successfully applied to potato (Johansson 1984). The effects of ethrel in wheat (Bennett and Hughes 1972) and rice (Hu et al. 1978) can also be seen in this context. The close parallels to the field of male sterility are obvious (Frankel and Galun 1977).

The effect of the donor plant growth conditions (including seasonal variation and plant age) on pollen plant formation in anther cultures had been known earlier (Sunderland 1971, 1978; Picard and deBuyser 1973; Foroughi-Wehr et al. 1976; Dunwell 1976; Keller and Stringam 1978). Their effect was mainly thought to influence inductor (hormone) levels in the anther wall, and the inductors were thought to be active after anther excision in the early days of anther culture (Dunwell 1976). The finding that these environmental effects specifically affect P-grain formation at a much earlier stage in vivo (meiosis), does, however, not mean that this is their sole effect on pollen plant formation. They also affect the level of inhibitory anther wall substances (Heberle-Bors and Reinert 1979; Heberle-Bors 1984a), which could be auxins (Dollmantel and Reinert 1980). In the same way, the specific effect of nitrogen starvation on sex balance and P-grain formation (Heberle-Bors 1983) should be distinguished from the general effect of minerals on plant, anther, and pollen vitality (Heberle and Reinert 1977; Heberle-Bors and Reinert 1979). Plant age does not affect P-grain frequency and pollen plant yield in pollen cultures of tobacco but only yields in anther cultures (Heberle-Bors and Reinert 1981), indicating again the involvement of anther wall substances in pollen embryogenesis (Pelletier and Ilami 1973).

A model for a phase change during the alternation of generations

The finding the P-grains are induced early in or shortly before meiosis (Heberle-Bors 1982b) fits in with a model of phase change during the alternation of generations

which has been developed by Bell (1970, 1979) and Dickinson and Heslop-Harrison (1977). During prophase of meiosis, the contact of the spore mother cells with the surrounding tissue becomes severed. Then, in the cytoplasm, the ribosomes are eliminated and the organelles dedifferentiate (Dickinson and Heslop-Harrison 1970a). This process is, however, incomplete. In micro- and megaspore mother cells, part of the sporophytic cytoplasm is protected from degradation by double or triple membranes, the encapsuled amount being greater on the female side (Dickinson and Heslop-Harrison 1977). The encapsuled cytoplasm thus contains polysomes with sporophytic m-RNA and non-dedifferentiated organelles. These structures, called sporophytic determinants, pass through meiosis and seem to affect subsequent spore development. They seem to be identical with the informational particles found by Bell (1979) which maintain or reactivate sporophytic competence in apogamic fern gametophytes. By analogy it is concluded that pollen embryogenesis is a form of apogamy (sporophyte formation via embryogenesis from somatic cells of the gametophyte). As with embryo sacs, sporophytic competence of pollen might result from a larger proportion of sporophytic determinants being encapsuled in the microspore mother cells. In normal male development, the sporophytic determinants seem to be involved in pollen wall patterning (Dickinson and Heslop-Harrison 1970b, Dickinson and Andrews 1976). It is therefore relevant that in the more recent ultrastructural studies on the early events of pollen embryogenesiss it has unequivocally been found that the wall around P-grains does not possess or lose the typical exine structure of gametophytic pollen and is considerably thinner (Sangwan-Norreel 1978; Rashid et al. 1981, 1982; Reynolds 1984). Physiological evidence for this model is still largely lacking. However, in cultured potato anthers, a four day pulse of actinomycin D did not impair pollen embryogenesis while cycloheximide did (Sopory 1979). This indicates the existence of preformed, stable m-RNA which might be produced by the described mechanism.

On the basis of this mechanism, the question of why P-grains are formed by plants can now be answered. P-grains represent a particular form of male sterility originating from a deviation from normal male development at the sporophytic-gametophytic transition in the microspore mother cells.

P-grain maturation and pollen stage

On a more general level, the formation of P-grains can be seen as a typical example of a terminal differentiation which has been better studied in animals (e.g. lymphopoesis or spermatogenesis). Heslop-Harrison (1980) has already pointed out that "the sporophyte-gametophyte transitions are in essence analogous with any other differentiation undergone in the plant body, albeit they are the most profound and happen the most abruptly".

Differentiation towards P-grain formation starts with a reversible determinative phase comprising the whole of meiosis (Heberle-Bors 1982b). By this, the specific competence for sporophytic development is acquired. Once acquired this competence is irreversible. Determination is followed by maturation. It starts with the early microspore stage and passes through the first pollen mitosis. An intermediate state of gametophytic gene expression followed by a period of gametophytic regression has been described by Bhojwani et al. (1973) and Dunwell and Sunderland (1974a, b, 1975). Rashid et al. (1981), however, claim that P-grains do not differentiate from the gametophytic pollen by way of regression, but that they are different structures to begin with and retain characteristics of the early microspores. The latter finding also applies to *Hyoscyamus niger* (Raghavan 1979) and *Datura innoxia* (Sangwan-Norreel 1978). In any case, in tobacco, it takes more than a week after the first pollen mitosis, depending upon the temperature, until pollen grains are formed which are devoid of most of their structural elements, only the two nuclei surrounded by a thin edge of cytoplasm being left. In this state, the pollen grain is ready for its first sporophytic division (premitotic pollen, P-grain). Maturation can take place in vivo, during chill treatment of excised flower buds or in cultured anthers (Heberle-Bors and Reinert 1981). Maturation is followed by the final phase, i.e. expression of competence, in this case, embryogenesis proper. Only at meiosis can the environment or hormones affect male gamete formation, from then on a system of internal, closed circuits maintains a strictly programmed development without interference from outside. Thus, once the susceptible phase (the switch-point) has passed, hormones or other factors are unable to change the programmed development. Therefore, factors operating later – be it chill or other treatments, including hormones, acting on microspores or pollen, shortly before or during culture – cannot increase the frequency of P-grains. They can only affect P-grain maturation or embryogenesis proper by supporting or inhibiting these processes. Most importantly, in cereals, conditions during P-grain maturation seem to control albino formation (Wang et al. 1978; Ouyang et al. 1983) which is a major drawback for pollen plant production in these species.

The time required for P-grain maturation differs not only within a species (Heberle-Bors 1984a) but also from species to species. *Datura innoxia* (Dunwell and Sunderland 1976a), henbane (Raghavan 1978) and potato (Dunwell and Sunderland 1973) seem to be the species with the shortest duration of P-grain maturation

(1–3 days after first pollen mitosis). Possibly, in these species, the intermediate phase of gametophytic gene expression is lacking so that the microspores represent true "direct spores" (Resende 1967) giving rise to the same generation as the one they are derived from. In cultured anthers of *Atropa belladonna,* in contrast, it takes 4–5 weeks for the first sporophytic divisions (Heberle-Bors 1980).

Until now, maturation of P-grains has not been seen as part of the process leading towards pollen embryogenesis. Differences in the duration of maturation have rather been treated in terms of appropriate pollen stage. From the very beginning of anther culture until today, success was only possible with immature anthers containing immature pollen. The optimal stage varies from species to species. Two basic optima have been distinguished. In one group of plants, the microspore stage is optimal, in the other group the pollen stage (Sunderland 1984). This distinction parallels the above mentioned distinction of species with long or short duration of P-grain maturation. The two expressions do, however, not mean exactly the same thing. In *Datura innoxia,* the optimal stage for anther excision is the pollen stage, while in henbane it is the microspore stage (Sunderland 1984). In both species, however, the pollen grains go through a normal first pollen mitosis (the one in vivo, the other in vitro) and embryonal divisions occur very rapidly thereafter. One should not forget that the optimal stage means anther stage although staging is performed on the basis of pollen development. The importance of this distinction becomes obvious when anther and pollen cultures are compared. For tobacco anther cultures, the optimal stage is around the first pollen mitosis to early binucleate stage. Before and after this stage, pollen embryos are also produced, but in lower and lower frequency the further away from the optimum. For pollen cultures, the optimal stage starts with the early binucleate stage (Heberle and Reinert 1977; Heberle-Bors and Reinert 1979). At earlier stages, no embryos are produced. However, at later stages, embryos are produced if the pollen is isolated and the P-grains separated from the normal pollen (Heberle-Bors and Reinert 1980; Heberle-Bors 1984a; Rashid and Reinert 1980, 1981). This indicates that the normal pollen grains and the anther wall produce inhibitory substances for the P-grains and young embryos. Obviously, developmental stage is a complex factor, composed of at least three parts: P-grain maturation, normal pollen grain maturation, and anther wall maturation. In different species, the timing of these three processes may be different (involving both gametophytic and sporophytic gene action), leading to the observable differences in optimal anther stage.

An important factor for pollen plant formation is pollen viability. Treatments which maintain viability of the P-grains are crucial for success and yield increases in pollen plant formation. A chilling treatment seems to be the most effective of these treatments (Nitsch and Norreel 1973) although failures have also been reported (Marsolais et al. 1984). Despite some controversy around the principal effect of chilling treatment (see "Nuclear routes" section), the experiments of Sunderland (1978) confirmed the earlier finding that the principal effect of chilling is on pollen viability (Duncan and Heberle 1976). Pretreatments such as water stress, anaerobic treatment (Imamura and Harada 1980a, 1981), or high humidity (Dunwell 1981) may also be effective by maintaining P-grain viability rather than by triggering new pollen grains into embryogenesis. Temperature, humidity, gas atmosphere and oxygen pressure are all factors known to affect normal pollen viability (Stanley and Linskens 1974) indicating that P-grain viability might depend on the same conditions as viability of normal pollen. It could therefore be that the bad performance of cereal pollen cultures (Wenzel et al. 1975; Heberle-Bors and Odenbach 1985) is caused by the generally bad viability of these pollens (Stanley and Linskens 1974).

It remains an open question whether the pollen grain is the primary target of these treatments, or the anther wall which secondarily affects pollen viability. Chilling has been found to delay anther senescence (Pelletier and Henry 1974; Sunderland and Roberts 1979) and increase total free amino acids in the anthers (Sangwan and Camefort 1978). Such changes in the anther wall possibly provide a micro-environment for the pollen which is more suitable for pollen viability at the beginning of culture. Alternatively, one can speculate that similar to chill hardening, chilling alters the composition of the pollen plasma membrane by the synthesis and incorporation of unsaturated fatty acids so that a more "fluid" membrane is produced (Lyons 1973). The resulting more fluid, adaptive membrane could enable the pollen grains to survive the stress of a changed environment (particularly water stress) much better than untreated pollen.

Embryogenesis proper

Nuclear routes to embryogenesis

The first embryonal divisions in the P-grains of many plants, including tobacco, usually start from the vegetative cell. One of the first noticeabale features is the formation of a fibrillar wall around the pollen protoplast and within the intine (Rashid et al. 1982). By strong cytoplasmic synthesis, redifferentiation of the organelles, and cell division, the P-grain and young embryo passes through a phase of proliferation until it reaches the globular stage. While in *Datura innoxia* the vegetative nucleus divides in the same plane as the microspore nucleus (Dunwell and Sunderland 1976a), the first embryonal division in tobacco can occur in different planes (Dunwell and Sunderland 1975), possibly

determined by the position of the generative cell (Rashid et al. 1982).

Apart from this standard pathway (A-pathway, Sunderland 1974), there exist quite a number of alternative routes, both within a species and differing from species to species. One possibility is that starting from a regularly developed P-grain with clearly differentiated vegetative and generative nuclei or cells, the vegetative nucleus divides without accompanying cell division. Thus, multinucleate pollen grains are formed which sometimes can be "rescued" by the inception of cell walls at later stages (Idzikowska and Mlodzianowski 1979). In those cases where the first embryonal divisions start already on the plant in situ they exclusively follow this route (Sunderland 1978; Heberle-Bors 1982a; Heberle-Bors and Odenbach 1985) indicating that cell wall formation is the prerogative of the in vitro process (Rashid et al. 1982). Another possibility is embryo formation from the generative cell or nucleus. This has been reported for henbane where it seems to be a regular feature (Raghavan 1976), and also for tobacco (Bernard 1971) and barley (Sunderland et al. 1979). A somewhat special route is the so-called B-pathway (Sunderland 1974). There, the first pollen mitosis is irregular and results in the formation of two equal-sized, vegetative-type nuclei. This equal division has caused a lot of controversy. Some people have claimed that it is the principal diverging point from normal gametophytic development towards embryogenesis, and that chilling or other treatments induce this diversion (Nitsch and Norreel 1973; Sangwan-Norreel 1978; Wilson et al. 1978). Although it seems that in some plants chilling does affect polarity of the first pollen mitosis (Sax 1935; LaCour 1949), it does not seem to play a decisive role for the induction of pollen embryogenesis. Chilling is effective not only before, but also and even more so, after the first pollen mitosis (Sangwan-Norreel 1977), pollen grains with equal nuclei are able to continue normal gametophytic development (Tanaka and Ito 1981), and no evidence for a chilling effect on the polarity of mitosis could be found in other studies (Duncan and Heberle 1976; Dunwell and Sunderland 1976b). Although chilling is effective in increasing pollen plant yield, it does so by maintaining viability of the cultured P-grains (Duncan and Heberle 1976, see above).

Pollen grains with two identical or two well-differentiated nuclei seem to be extremes of a continuum. In tobacco, and more so in wheat, all kinds of intermediate states of nuclear condensation of the generative nucleus can be found (Heberle-Bors, unpublished results). This could indicate that the degree of polarity in the microspore determines the degree of cytoplasmic partitioning at the first pollen mitosis, and that the amount of cytoplasm in the two cells determines the degree of nuclear condensation (Sangwan-Norreel 1978). Interesting as this finding may be, it has to be pointed out again that the developmental decision for pollen embryogenesis is taken before the first pollen mitosis. Thereby, embryogenic competence can be conveyed to both of the cells, and the diversity of routes towards embryogenesis seems to be a reflection of the relative unimportance of these routes. In one aspect, however, cytoplasmic partitioning may be important. In henbane, where pollen embryogenesis starts from the generative cell, this cell contains an appreciable amount of cytoplasm (Reynolds 1984). This indicates that structures in the cytoplasm are in some way important for embryogenic induction. These structures should be identical with the sporophytic determinants formed at meiosis by the above mentioned, postulated mechanism. Interestingly, the generative cell in P-grains of henbane contains plastids while in normal pollen grains it does not (Reynolds 1984). This coincides with the finding that cytoplasmic genes are involved in P-grain formation (Heberle-Bors and Odenbach 1985, see "Genotypic control" section).

Embryo abortion and culture medium

An intriguing aspect of anther and pollen culture is that the frequency of P-grains and early pollen embryos can be quite high (up to 15–20% in tobacco) but that the final yield of plantlets is lower by about two orders of magnitude (Horner and Mott 1979; Heberle-Bors and Reinert 1979; Heberle-Bors 1984a). Embryos can abort at many, presumably at all, stages of embryogenesis. Most embryos abort at a very early stage. Abortion is, in most cases. associated with the formation of vacuolated, callus-like cells. In early stages, all cells of the embryo can be transformed in this way. At later stages, only the cells at the surface are transformed. Embryos at the globular and later stages with a normal appearance can, however, also be found in anther and pollen cultures which do not develop further. In some species, particularly in cereals, aborted embryos can be rescued. In these species, hormones in the culture medium maintain cell divisions in the aborted embryos. Embryogenic calli are formed which can regenerate adventitious embryos after manipulation of hormone balance but even there, the vast majority of multicellular structures die (Sunderland 1984).

This high abortion rate can be attributed either to genotypic factors (lethal genes, Henry et al. 1984) or it could mean that the cultural requirements for pollen embryos are still inadequately understood. As support for the latter assumption, activated charcoal can increase the number of normal embryos and plantlets in tobacco without affecting the total number of pollen embryos (normal plus abortive ones, Heberle-Bors 1980). It acts by adsorbing inhibitors from the agar (Wernicke and Kohlenbach 1976) and possibly from the anther wall (Weatherhead et al. 1978, 1979) and from advanced embryos (Johansson 1983). Its effectiveness depends upon the type of charcoal used (Heberle-Bors 1980). Care has to be taken when particular iron chelates are used in combination with charcoal. Sequestrene 138 is completely adsorbed by charcoal re-

sulting in a complete inhibition of pollen embryogenesis at the globular stage (Heberle-Bors 1980).

Several other factors have been identified which increase pollen plant yield. These factors have been reviewed sufficiently in the recent reviews (Maheshwari et al. 1980, 1982). Here, emphasis is laid upon more recent developments and the possible involvement of these factors in embryo abortion.

Although different mineral formulae have been applied for anther and pollen culture (see Maheshwari et al. 1980, 1982), it appears that the Nitsch formula (1969) is the most frequently used. The situation is somewhat different in cereals where formula based on the ones of Murashige and Skoog (1962) and Miller (1963) are more widely used. Recent work in tobacco, however, has shown that even in the classical "Nitsch plant", the minerals of Miller are superior when pollen cultures are performed (Heberle-Bors 1984a, in preparation). A particularly important ion for pollen embryogenesis is the proton. So far, a pH of 5.8, the traditional pH, set before autoclaving, has been used in anther cultures. By decreasing proton concentration (pH 6.8–7) in filter-sterilized media, however, considerable yield increases have been achieved in pollen cultures (Rashid and Reinert 1980, 1981; Heberle-Bors 1984a). Interestingly, the pH effect made it possible to remove glutamine and asparagine from the medium. Amides were once thought to be essential for pollen cultures (Nitsch 1974a; Wernicke and Kohlenbach 1977; Heberle-Bors and Reinert 1980). Another particularly important ion is Fe^{2+}. Although required only in minute quantities (10^{-5} to 10^{-4} Mol/l), it controls a major step in pollen embryogenesis, i.e. establishment of the basic body plan (transition from the globular to heart-shaped stage, Nitsch 1969, 1972; Havranek and Vagera 1979; Heberle-Bors 1980). It has been proposed that the chelator acts in a hormone-like manner (Gupta and Maheshwari 1970) since cytokinins seem to mimic chelator effects (Sopory and Maheshwari 1973). From the work of Vagera et al. (1979), however, it appears that kinetin cannot substitute for EDTA, and it has been proposed that the chelate complexes are effective by their different binding stability for iron (Heberle-Bors 1980). Unfortunately, studies on the effect of Fe-chelates have not been performed with somatic embryogenesis, so it is not possible to generalize this effect. In this context, another factor should be mentioned which controls the establishment of the basic body plan. The orientation of the embryo axis seems to depend upon the site of exine rupture (Kohlenbach and Geier 1972; Heberle 1976; Imamura and Harada 1980a). Possibly, a gradient in the embryo set up by differential uptake and/or leaching-out of substances could be the active principle.

As a carbohydrate source, 2–3% sucrose has been most frequently used (also in tobacco). In some species, especially in cereals, however, higher concentrations have been found beneficial (Clapham 1977). Recent work with tobacco pollen cultures has shown that high sucrose concentrations are also beneficial in this model species in early stages of pollen embryogenesis (Heberle-Bors 1984a, in preparation). Rashid and Reinert (1981) could not reproduce this effect but only concentrations up to 4% were used in their study. At later stages of embryogenesis, sucrose has to be reduced in pollen cultures. This is similar to results found in pollen cultures of *Brassica napus* (Lichter 1982) and in anther cultures of *Brassica campestris* (Keller et al. 1975) and potato (Sopory 1979). It appears from this and other findings (minerals, pH, see above) that in tobacco, and possibly in other species, the anther wall veils many effects of the culture medium so that common features of pollen embryogenesis in different species cannot be recognized.

In such well studied plants as tobacco, *Datura innoxia*, henbane, and a few others, embryos develop in a medium without added hormones. The hormones can even be inhibitory (Nitsch 1974b; Raghavan 1978; Taguchi and Mii 1982). In other cases, one or the other hormone has been found necessary. However, in most of these cases, the studies are not sufficiently detailed to enable a judgement on the relative efficacy of these hormones. The inhibitory effect of 2,4-D in henbane (Raghavan 1978) can be seen in close analogy to somatic embryogenesis in carrot. There too, the presence of 2,4-D inhibits embryogenesis and leads to callusing while the removal of auxin allows maintenance of embryonal development (Reinert 1958, 1959; Kohlenbach 1977). In studies on somatic embryogenesis, it could recently be shown that cytokinins do not act as specific inducers of embryogenesis, but act merely as cell division factors required for a limited period until the embryo becomes hormone-autotrophic (Ernst and Oesterhelt 1984; Ernst et al. 1984). A similar view might apply to pollen embryogenesis. On the basis of embryogenic competence of the P-grains, hormones may be active in starting and maintaining cell division. In some plants – possibly in many, once the need for hormones is only critically tested in pollen cultures – the P-grains might be hormone-autotrophic from the beginning. In others, it may take some time to acquire hormone-autotrophy, and hormones have to be supplied exogenously. In the former plants, many embryos abort, and abortion may be related to insufficient endogenous hormone synthesis. Over-optimal hormone concentrations can also occur (Dollmantel and Reinert 1980) although hormone levels in pollen grains and whole anthers have to be distinguished. In any case, there seems to be a need for critical studies on the effect of hormones on pollen embryogenesis, particularly on the ratio of cell division to cell enlargement (nucleo-cytoplasmic ratio) during early development. At later stages of embryogenesis it seems to be a regular feature that exogenous hormones are no longer required.

Genotypic control

With regard to developmental control of pollen plant formation, one would generally expect that the environment in interaction with genotype would control all phases of development. In fact, it was found in tobacco that genotype did control P-grain frequency under standard growth conditions but particular en-

vironmental factors and their degree of interaction with genotype could also be isolated (Heberle-Bors 1984a). Essentially the same results have been reported for *Brassica napus* (Thurling and Chay 1984) although these results were based upon the frequency of multicellular pollen units in vitro instead of P-grains in vivo. More specifically, it was found that cytoplasmic genes (cytoplasmic male sterile (cms-) lines in wheat) also affect P-grain induction (Heberle-Bors and Odenbach 1985). In the following phase of P-grain differentiation, genotype controls the time required to complete P-grain maturation (see "P-grain maturation and pollen stage" section).

During the final phase, embryogenesis proper, genotype affects the performance of anther cultures. It had been known for some time that the anther wall contributes to pollen embryogenesis by providing both beneficial (Pelletier and Ilami 1973; Nitsch and Norreel 1973; Nitsch 1974a) and inhibitory substances (Heberle-Bors and Reinert 1979). A comparison of tobacco genotypes revealed that genotype and plant growth temperature interact in the production of an inhibitor in the anthers (Heberle-Bors 1984a). In anther cultures, this inhibitor annihilated the yield-increasing effect of a higher P-grain frequency induced by low plant growth temperature. By carrying out isolated pollen cultures, however, the inhibitor could be removed.

The effect of genotype has been known since the early days of pollen plant production (Nitsch 1969; see also Maheshwari et al. 1980). However, only in recent years has it been studied more intensively. In barley, four independently and differently inherited traits have been distinguished (Foroughi-Wehr et al. 1982) of what has been called "tissue culture ability" (Wenzel 1980): "callus induction", "callus stabilization", "plantlet regeneration", and "albino versus green plantlet formation". A similar distinction has been seen in wheat, lacking only "callus stabilization" (Lazar et al. 1984a, b). In triticale, Charmet and Bernard (1984) have also used "callus (embryo) induction", but combined "plantlet regeneration" and "green plantlet formation". Because of the dominating effect of genetic factors over cultural factors, these authors suggest a breeding strategy for pollen plant production as opposed to a physiological strategy. Such a breeding strategy can lead to recombinant lines in which the specific genes for efficient haploid production are accumulated. It can, however, also introduce complications into a breeding programme which could annihilate the time-saving effect of doubled haploids.

The genotypic effects found during studies conducted by this author (Heberle-Bors 1984a; Heberle-Bors and Odenbach 1985) can partly be made to agree with the above mentioned traits. "Callus or embryo induction" could be compared with P-grain frequency and induction rate. This is, however, only possible if "callus induction" (calli per hundred anthers) is converted into calli per hundred pollen grains (see "Anther and pollen culture" section). While Foroughi-Wehr et al. (1982) and Charmet and Bernard (1984) did not consider donor plant environment interaction in this trait, Lazar et al. (1984a) did find such interaction. This is comparable to the findings on P-grain frequency in tobacco (Heberle-Bors 1984a) and on multicellular unit frequency in *Brassica napus* (Thurling and Chay 1984). "Callus stabilization" and "plantlet induction" are traits which have to do with the organogenetic approach to plantlet regeneration. With direct embryogenesis, these two traits can be considered as one (Lazar et al. 1984a, b; Charmet and Bernard 1984) and could be compared with embryo abortion (success or failure, respectively to complete embryogenesis). One conclusion from this is that the genetic traits discovered by different authors depend upon the experimental procedure employed, and this reflects the above mentioned general statement that any developmental step has a genetic basis.

Summarizing this discussion on the genetic traits identified by different authors, it appears that the P-grain concept with its clear distinction of donor plant and in vitro response provides many more specific traits for the synthesis of recombinant haploid producer lines than the mere formal "tissue culture" approach adopted by many authors. At least in cereals, where P-grains can be easily detected, there is no reason whatsoever to neglect the P-grain concept in future work.

A controversial situation exists at present with regard to the involvement of cytoplasmic genes in pollen plant formation. In some experiments with reciprocal crosses, maternal effects could be found but these were not found in others (Picard et al. 1978; Foroughi-Wehr et al. 1982; Bullock et al. 1982; Lazar et al. 1984b; Charmet and Bernard 1984). Similarly, in some cms-lines, higher pollen plant yields could be found as compared to those found in male fertile lines in some cases (Ling et al. 1978), but not in others (Picard and deBuyser 1973; Misoo and Mitsubayashi 1982; Misoo et al. 1984). This coincides with the finding that in wheat certain cms-lines produce high frequencies of P-grains, while other cms-lines do not (Heberle-Bors and Odenbach 1985). Foroughi-Wehr and Friedt (1984), in contrast, maintain that "the transfer of androgenetic responsiveness to F_1-hybrids of wheat, barley and probably other cereals does not depend on the source of cytoplasm. It rather depends on nuclear genes . . .". In fact, the author's work showed that pollen embryogenesis does not depend upon the source of cytoplasm since highly embryogenic cms-lines could be found with all cytoplasms included in the screening (Heberle-Bors and Odenbach 1985). On the other hand, not all cms-lines with one particular cytoplasm were

highly embryogenic, and this indicates that, in addition to cytoplasmic genes, other genetic (i.e. nuclear) factors must be involved. Similarly, in triticale, maternal effects on pollen plant yield could be found with both cytoplasms used (*Triticum aestivum* and *T. timopheevi,* Charmet and Bernard 1984). A particular role for the *T. timopheevi* cytoplasm (Picard et al. 1978) or any other cytoplasm can therefore be ruled out, and it appears that the cytoplasmic contribution to P-grain formation is common to all cytoplasms. In conclusion, P-grain formation seems to be controlleld by an interaction of cytoplasmic and nuclear genes which is further modified by environment.

Separate from the involvement of cytoplasmic genes in P-grain formation is their involvement in albino pollen plant formation of cereals. Reciprocal crosses in wheat and triticale suggest maternal effects (Bullock et al. 1982; Charmet and Bernard 1984). Albinism seems to be based on deletions of plastid DNA (Day and Ellis 1984) and deficiencies in plastid ribosomal RNA and proteins (Sun et al. 1979). Besides genetic factors, physiological factors are also involved (Wang et al. 1978; Ouyang et al. 1983). The basic problem whether the deletions are a result of pollen development in vivo or of callus or embryo development in vitro has not yet been resolved (Day and Ellis 1984) nor has the question whether they are related to P-grain development as opposed to normal pollen development.

As a general conclusion from the genotypic effects, it appears that pollen embryogenesis should be seen as a product of evolution. The question then arises at which point in evolution did the capacity for pollen embryogenesis appear. This question cannot be resolved yet, but it appears that in essence this is the question of a continuity of male apogamy from the ferns to the higher plants and of the appearance of male sterility.

As an evolutionary product, pollen embryogenesis is open to variation. This variation creates problems for the applicability of pollen haploids to dihaploid breeding. The breeders want to obtain haploids from any genotype. It has, however, to be accepted that P-grain formation can be manipulated only within the limits imposed by genotype. It is therefore necessary to utilize as efficiently as possible the genotypically determined range of environmental plasticity for P-grain formation and to allow the sometimes low number of P-grains to develop undisturbed. On the other hand, the rational for the synthesis of recombinant haploid producer lines lies basically in this limitation.

A paradigm change

The new view of pollen embryogenesis presented has not been easily accepted and still meets with resistance. The indeterministic totipotency dogma of plant tissue culture still demands that a cultured plant cell can be manipulated at will provided the right trigger can be found. The new view instead holds that in vitro cultured cells may not be totipotent in the sense that certain genetic information may be irreversibly blocked or even lost, and that present-day culture media ingredients are not able to break these blocks. Light, temperature, ions or hormones may affect development only in early stages of a developmental pathway, by acting on an endogenously determined reaction system with limited and specific developmental choices. These choices in turn are determined by both ontogenetical and phylogenetical conditions. The same view also holds for other in vitro systems such as flower formation from cultured tobacco cells, regeneration from cereal mesophyll protoplasts, regeneration from mature woody plants, or habituation (Heberle-Bors 1984 b, c).

Similar to tissue culture, the field of spore development is also governed by the totipotency concept. The model we present was developed by Bell (1970, 1979) and Dickinson and Heslop-Harrison (1977). It can be called a deterministic model since spore development is thought to be determined during sporogenesis. The presently more accepted view, in contrast, is an indeterministic one. It is based upon developmental neutrality (this is what is generally meant by and mixed up with the term totipotency) of the spores, and spore development is thought to be controlled by the factors operating once the spore has been formed, i.e. at the time of spore germination (Vasil 1973; Vasil and Nitsch 1976). This view obviously has influenced many workers on pollen embryogenesis. For example, Sunderland (1984) summarized his experiences in a model in which the inductive events occur around microspore division although he concedes that "division of the gametophytic cells is ... an expression of sporophytic influences carried over from the diplophase through meiosis". Even Rashid et al. (1981) who acknowledge P-grain predetermination by the growth conditions of the donor plant maintain that tobacco microspores pass through an indeterminate stage and that chilling treatment is necessary as an inductive agent.

Other switch-points?

One argument against the new view is that there exist species where no correlation can be found between in vivo P-grain frequency and the frequency of in vitro pollen embryos. Possibly in some species, P-grains are not easily detectable due to staining difficulties. Or, as in tobacco, the P-grains quickly die in vivo (Heberle-Bors 1982 a). In this case, one should count the total number of dead grains (Heberle-Bors 1984 a). An inhibitor can also veil this correlation in anther cultures, but not in pollen cultures (Heberle-Bors 1984 a). In still other species, no obvious morphological differences between normal and P-grains may occur – only physio-

logical ones (Thurling and Chay 1984). Although it appears that, as with spermatogenesis in animals, many plants do not allow male gametogenesis to depend strongly upon environmental conditions, since it is a too essential part of the life cycle, it may be possible that in some species gametogenesis is not so strictly programmed as in tobacco, and that there exist further developmental switch-points. In this case, however, one would expect that on the basis of the gametophytic gene programme being already switched on, different gametophytic structures are formed. Possibly, the formation of pollen embryo sacs, i.e. female gametophytes (Nemec 1898), could result from such an event. Nevertheless, the possibility remains that natural selection "invented" more than one switch-point for the return into the sporophytic gene programme. In *Datura innoxia* and henbane, no P-grains in vivo have been reported yet and sporophytic divisions start very quickly after the first pollen mitosis. This could indicate that the sporophytic gene programme is only a little repressed in these species and can be easily reactivated by the trauma of excision and inoculation. It is, however, suspicious that in these species, as in tobacco, only a small number of pollen grains are embryogenic (maximal 1% in henbane, Reynolds 1984; 2–3% in *Datura metel,* Sangwan 1983). If these spores were neutral or only little repressed, the frequency of P-grains should be under better experimental control once they are separated from the plant. These considerations, however, do not alter the concept that the basic developmental decision – gametophytic or sporophytic – is taken during sporogenesis at meiosis. In species with obvious pollen dimorphism, the inductive trigger may be strong enough (amount of sporophytic determinants) to switch pollen development in vivo. In species without obvious pollen dimorphism, the P-grains might require the trauma of excision for the release of their predetermined potency. The rapidity with which P-grains in henbane can be distinguished from normal grains (1 h after anther excision, Reynolds 1984) is indicative of such a subthreshold level. Further work will have to show whether in these plants, P-grains can be identified already in vivo by more refined techniques.

A more direct challenge to the new view has come from the observation that in barley P-grains have been found in the centre of the anther, while pollen embryos have been found only at the periphery of cultured anthers (Idzikowska et al. 1982). However, the pollen grains in the centre of these anthers are probably not P-grains, but retarded normal pollen grains, i.e. microspores, since they are uninucleate and contain a vacuole. But even if they were P-grains, these findings contradict another study on barley that both P-grains in situ and embryos in cultures anthers are concentrated in the basal part of the anther (Dale 1975). In henbane, P-grains can be found in the centre and at the periphery while embryos are again confined to the periphery (Raghavan 1978). Possibly, P-grains at the periphery benefit from the nutrients coming from the medium and the tapetum while the P-grains further inside are cut off from these nutrients. This seems to be very likely in big anthers with tens of thousands of pollen grains as in henbane or tobacco. However in cereals such as barley, where it is sometimes difficult to distinguish more than one layer of pollen grains along the tapetum, this argument does not hold. There, the pollen embryos developing from centrally located P-grains probably push through the degenerating normal pollen grains so that they appear to originate from pollen grains at the periphery. Such displacements, although in the opposite direction, have been described in henbane (Ranghavan 1978). In any case, these findings point to the switch from anther to pollen culture in order to give the centrally located deprived P-grains an equal chance to develop into embryos.

Anther versus pollen culture

Another reason for the resistance towards the new view has to do with the success of anther culture over the competing technique of pollen culture. Anther cultures are easy to perform and the anther wall provides a physical and chemical environment supporting pollen embryogenesis to a sufficient degree in many species. The facility of performing anther cultures therefore suggested a strategy of culturing high numbers of anthers rather than to increase their efficiency. Pollen cultures, on the other hand, still pose problems. Even in tobacco, difficulties have been encountered (Horner and Street 1978) in trying to reproduce the original version of pollen culture which involved preculture of the anthers (Nitsch 1974a; Reinert et al. 1975). In the meantime, new variations of this technique have been developed. One way is to subject excised young flower buds to a chilling treatment and to isolate and separate the P-grains thereafter (Rashid and Reinert 1980, 1981). This variation utilizes the viability-increasing effect of the chilling treatment (Nitsch and Norreel 1973; Duncan and Heberle 1976). Another variation is to isolate the pollen directly from the plants. There are two different procedures. Either relatively young pollen is isolated (Imamura et al. 1982; Gandhimathi 1982), or mature, ready-to-divide P-grains are isolated and separated from the relatively mature normal pollen (Heberle-Bors and Reinert 1980).

In such pollen cultures, two parameters are sufficient to determine the effect of a given factor. Induction rate is the frequency of pollen grains which have formed an embryo (Heberle-Bors 1980). A pollen embryo is defined as a pollen grain with at least two (A-pathway) or three (B-pathway) vegetative-type nuclei or cells. Induction rate should compare with the predetermined number of P-grains provided all P-grains stay alive in culture. The second parameter is abortion rate. With these two parameters the effect of any treatment in pollen cultures can be evaluated and is related to the unit which actually produces the embryo, i.e. the pollen grain.

In anther cultures, more and different parameters based on quite sophisticated calculations are in use (Sunderland 1974; Dunwell 1976). By this, very impressive "yields" can be obtained reaching 100% induction frequency simply by using not the pollen grain as unit of yield assessment but the anther. Superficially, it seems to be justified to use the anther as the unit when anther cultures are performed. However, it is the pollen grain which produces the embryo, and yield assessment on an anther basis totally abstracts from the number of pollen grains per anther. Yields calculated in this way can therefore not be compared with yields based on the pollen grain as unit because the number of pollen grains per anther are usually not mentioned in publications. It developed, therefore, that cereals are regarded as low-yielding species. If one, however, knows that a wheat anther contains only 2–4,000 pollen grains, whereas a tobacco anther ten times more (40,000), and if one calculates the responsiveness on the presently obtained yields, the differences are minor. In the experiments of Henry et al. (1984), 1,280 advanced embryos have been obtained from 15,300 anthers (8.4%). Division by the number of pollen grains per anther (2,000) yields 0.0042%, wich is in the range of tobacco (0.008% from untreated donor plants, Heberle-Bors and Reinert 1979). Also on the basis of P-grain frequencies in vivo, tobacco and wheat show similar yields (Hadwiger and Heberle-Bors, in preparation). It has to be admitted that the figures thus obtained are very low and not very impressive. But they might open the eyes to a realistic view of the possibilities to increase pollen plant yields, and may help to reinforce the shift of efforts towards the donor plants and towards pollen culture.

Summary and outlook

In summary, pollen embryogenesis seems to consist of three distinct phases – P-grain induction, P-grain maturation, embryogenesis proper – in which development is controlled by different factors. Attempts to increase pollen plant production should therefore adopt an integrated strategy comprising studies on optimal conditions in each of the three phases.

Beyond this direct applied aspect further-reaching aspects appear. 1) Pollen embryogenesis is a model system approximating embryo sac embryogenesis in vivo (zygotic, parthenogenetic, apogamic) much closer than somatic embryogenesis in vitro. In fact, it is a homologous system. The processes from the acquisition of sporophytic competence to embryogenesis proper seem to occur in essentially the same way in both systems. While, however, only few megaspores, embryosacs, and embryos are produced, enclosed in the complex structure of the ovules and seeds, microspores and pollen grains are produced in abundance in the anthers, and P-grains and pollen embryos can be cultured isolated from surrounding tissues. This should make pollen embryogenesis a highly suitable experimental system for many aspects of plant gametogenesis and embryogenesis. 2) The model sees plant embryogenesis convergent to animal embryogenesis without neglecting the plant-specific alternation of generations (Heberle-Bors 1984d). In animals it is established that preformed maternal messengers in the egg control early embryogenesis (Gurdon 1974). The assumed sporophytic determinants could represent the respective analogous structures in plants. 3) Eradication of sporophytic influences during sporogenesis combined with the transmission of sporophytic determinants are the two basic events of reprogramming of somatic cells towards gametogenesis and embryogenesis, and this is identical with what has been called rejuvenation. Lack of rejuvenation is at present the major obstacle for in vitro regeneration and cloning of mature elite forest trees and probably of other plants. By studying the natural process of rejuvenation during meiosis, one should get some insight in its regulative factors which could then help to develop methods to induce rejuvenation. 4) For clonal propagation and genetic engineering, embryogenesis is the desirable way of regeneration, superior to the shoot-root sequence from callus. Only through embryogenesis can the relation "one cell – one organism" be established and genetic aberrations (somaclonal variation) be minimized. In somatic embryogenesis, the "embryo mother cells" have still not been identified or isolated, and therefore, some uncertainty exists about the inductive and regulatory factors. With pollen embryogenesis this uncertainty does not exist, and it is the only in vitro system with which embryogenesis from single cells can be achieved in practically all higher plants, including cereals.

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