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FACTORS INVOLVED IN INITIATION OF SOMATIC EMBRYOGENESIS IN CEREAL TISSUE CULTURE

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Recent data concerning somatic embryogenesis in cereal tissue culture are reviewed. A comparison between the culture of immature explants (embryo and inflorescences) and both mature embryo and parts of young seedlings is made. Somatic embryogenesis in the culture of immature explants proceeds from the explant cells which were embryogenic before explanting into culture, whereas a certain degree of redetermination of cells of more mature explants is necessary before induction of embryogenic callus in the culture of either mature embryo or parts of young seedlings. Embryogenic cells in the culture of more mature cereal explants do not seem to originate directly from explant cells, but the conversion of cells of preceding non-embryogenic callus to embryogenic ones is likely source of embryogenic callus. Physical isolation of a certain cell from its neighbourhood seems to be the most critical factor enabling somatic embryogenesis. Different stimuli, by which the escaping of the cell from control of the tissue as a whole can be achieved, are discussed (breaking of plasmodesmata, necrosis of surrounding cells, stress conditions and senescence).

Introduction

The normal pattern of plant development starts after zygote formation although the capacity to produce the whole plant does not reside in the zygote alone and it may well persist, even though suppressed, in almost any living cell of the plant body. Thus, somatic plant cells are

totipotent (although totipotency has not been demonstrated for all plant cells) indicating that plant cells can undergo differentiation without loss of the initial genetic potentialities of the zygote from which they were ultimately derived. Under appropriate stimuli somatic plant cells can display different aspects of their genetic potentialities, including their potentiality to initiate somatic embryoids which can develop into differentiated plants (the process which is known as somatic embryogenesis).

Somatic embryogenesis occurs naturally in many species (for rev. see Vasil and Vasil 1980). In addition, a vast body of data has been collected about somatic embryogenesis as a way of plant regeneration from *in vitro* tissue culture. The scope of this article is limited to somatic embryogenesis in cereal tissue culture as a subject where significant progress has recently been made.

Comparison between immature and more mature tissue explants

a) *Callus types in cereal culture*

There are two types of callus tissue which can be distinguished in cereal culture. One type is comprised of small meristematic cells with dense cytoplasm and large nuclei with enlarged nucleoli (embryogenic, E, callus). Embryogenic cells are the source of somatic (non-zygotic) embryos (embryoids) which germinate into green plants.

Other type of callus exibits a minor regenerative capacity (non-embryogenic, NE, callus) and consists of large, highly vacuolated parenchyma cells (Vasil and Vasil 1982a, b, Nabors et al. 1983, Botti and Vasil 1984, Heyser et al. 1985, Magnusson and Bornman 1985).

b) *Immature tissue explants*

Very young or immature explants are usually used for E callus initiation (immature embryos and young inflorescences) whereas differentiated, mature cereal tissues are recalcitrant to tissue culture manipulation.

The explant most frequently used for E callus induction is immature embryo (Thomas et al. 1977, Vasil and Vasil 1981, 1982b, Heyser and Nabors 1982a, Lu and Vasil 1982, 1985, Lu et al. 1982, 1983, 1984, Ozias-Akins and Vasil 1982, 1983a, b, Heyser et al. 1983, Thomas and Scott 1985, etc.). Cells comprising immature embryo is considered to be embryogenic i.e. the development of somatic embryoids proceeds from cells which were embryogenic before explanting into culture (direct embryogenesis from pre-embryogenic determined cells, PEDC's, terminology of Sharp et al. 1980).

Because the tissues of immature embryo are comprised of embryogenic cells, the conditions of culture are the factors which make particular tissue and/or cell proliferate into E callus and somatic embryoids. E callus is most frequently originated in scutellum if the immature embryo was placed with embryo axis in contact with agar medium and scutellum exposed (Thomas et al. 1977, Vasil and Vasil 1981, 1982b, Lu et al. 1982, 1983, 1984, Ozias-Akins and Vasil 1982, 1983a, Lu and Vasil 1985, Thomas and Scott 1985 etc.). If the scutellum of intact immature embryo is placed in contact with agar

medium, other tissues of immature embryo take place in E callus formation (nodal region: Vasil et al. 1983 in maize, Heyser et al. 1985 in wheat; or epiblast: Ozias-Akins and Vasil 1983b in wheat). Generally speaking, scutellum of the immature embryo exibits the capacity for E callus formation in a very limited period of embryo development (in duration of 24—48 h. Armstrong and Green 1985, Kamo et al. 1985) which usually falls about the twelfth day after anthesis, depending on species and variety. Cultivation of younger or older (mature) embryos, generally, does not yield E callus from scutellum cells (Ozias-Akins and Vasil 1982, Krumbiegel-Schroeren et al. 1984, Lu et al. 1984, Thomas and Scot 1985)

Similar observations have been made for inflorescence explants too. Only those in which floral primordia were just being initiated were found to be the best source of E calli, whereas the younger as well as the older inflorescences were found to be unsuitable (Brettell et al. 1980, Vasil and Vasil 1981, Lu and Vasil 1982, Ozias-Akins and Vasil 1982, Wang and Vasil 1982, Rangan and Vasil 1983, Botti and Vasil 1984, Boyes and Vasil 1984, Chu et al. 1984, Fedak 1985).

An understanding of the factors causing such link between developmental stage and embryogenic capacity may eventually lead to the elucidation of the control of plant differentiation.

c) *More mature tissue explants*

Mature embryos and parts of young seedlings have also been used for establishing E callus (Thomas et al. 1977, Wernicke and Brettell 1980, Haydu and Vasil 1981, Lu and Vasil 1981a, Heyser and Nabors 1982a, b, Wernicke et al. 1981, 1982, Botti and Vasil 1983, Nabors et al. 1983, Fatokun and Yamada 1984, Rengel 1984, 1987a, b, Weigel and Hughes 1985, Rengel and Jelaska 1986a, and others). Those explants are comprised of tissues and cells of various levels of differentiation which is closely related to their ability to produce E callus. A certain degree of redetermination (dedifferentiation) of those cells might be necessary in order to initiate the development of somatic embryoids from them (Rengel and Jelaska 1986a) (indirect embryogenesis from induced embryogenically determined cells IEDC's, terminology of Sharp et al. 1980).

Although there have been no reports on extensive histological investigation of E callus formation in the culture of mature cereal tissues at this time, some observations indicate that meristematic tissue of shoot apical region is the source of E callus, whereas the scutellum of cultured mature embryos has never given rise to somatic embryoids, regardless of orientation to the agar medium (Botti and Vasil 1983, Heyser et al. 1985, Rengel 1987a, b). However, it should be mentioned that Thomas et al. (1977) observed embryoid formation from scutellum of mature *Sorghum* embryos and Nabors and his collaborators mentioned the same observation for mature rice scutella (cit. after Heyser et al. 1985). In either case, no histological evidence was presented.

At this time it is not possible to induce E callus formation from mature, fully differentiated tissues of cereals. The fact that only meristematic regions proliferate into E callus points out that it is easier to maintain the high rate of mitotic divisions in meristematic cells than to

re-start divisions in already differentiated cells. Accordingly, in the course of differentiation of plant cells there is a loss of competence for responding to *in vitro* manipulation (which does not necessarily include loss of totipotency). Whether this problem may be overcome by changing *in vitro* growing conditions, remains to bi answered.

The origin of E callus and somatic embryoids

In the culture of immature and more mature explants somatic embryoids usually develop from E callus and very rarely (Dunstan et al. 1978, Lu and Vasil 1985) directly from cells of the scutellum of immature embryo.

The question regarding unicellular or multicellular origin of somatic embryoids is beyond the scope of this article and was discussed elsewhere (Haccius 1978, Wernicke et al. 1982, Vasil 1983, Williams and Maheswaran 1986).

After E callus was formed, the development of somatic embryoids is likely to be the same regardless of the starting explant. However, differences between immature and more mature explants exist in the way of establishing E callus. E callus arises directly from immature explants i. e. embryogenic cells of explant undergo continuous divisions yielding E callus. From induced E callus (or proembryogenic cell complex) somatic embryoids orginate in different ways (Lu and Vasil 1981b, Vasil and Vasil 1981, Wernicke et al. 1982). On the other hand, only NE callus could be seen on more mature explants in the primary culture (except in the culture of either mature seed or root sections of some rice varieties; Siriwardana and Nabors 1983, Raghava Ram and Nabors 1984, Abe and Futshuara 1985). Additional subculturing of induced NE callus (usually on the same induction medium as the one used in the primary culture) results in the appearance of E callus (Heyser and Nabors 1982a, Botti and Vasil 1983, Heyser et al. 1983, 1985, Nabors et al. 1983, Chandler and Vasil 1984, Abe and Futsuhara 1985, Weigel and Hughes 1985, Rengel 1987a, b, Rengel and Jelaska 1986a).

The question concerning the origin of E cells in the NE callus can be raised. One of the speculations is that E cells come out from explant cells but induced E cells are »suspended« and masked in the mass of fast growing NE callus. Such E cell »pockets« could give rise to E callus and somatic embryoids in certain culture conditions (Botti and Vasil 1983). This is consistent with the postulation of non-convertibility of NE to E cell populations (Street 1979, Vasil 1983). On the other hand it can be proposed that only NE cells come out from explant cells and, during subsequent subculturing, some NE cells undergo dedifferentiation up to the embryogenic stage and they yield E callus (Nabors et al. 1983, Rengel 1984, Heyser et al. 1985, Rengel and Jelaska 1986a). In the latter postulation, conversion of NE cells to E ones is the crucial point. The proposed unequal division of highly vacuolated NE cells, yielding both small cell with meristematic character and large, vacuolated parenchyma cell, has not been histologically demonstrated in the culture of mature cereal tissue at this time but was observed earlier in somatic embryoid development from single carrot cell (Raghavan 1976), in division of parenchymatic cells of cultured mature lemmon fruit sacs (Kordan 1977) as well as during normal non-somatic) embryogenesis

of many species (Maheshwari 1950) and in stamen-hair initiation in *Tradescantia* (Mericle and Hazard 1980). Moreover, continuous E callus production in long-term NE callus cultures of barley (aged up to 21 months), where only NE callus pieces were repeatedly subcultured whereas E callus was always removed during transfer, could be set in support to postulation of conversion of NE cells to E ones. Maintaining E cells in concealment in a mass of NE callus (if they were raised directly from explant cells) is unlikely to be valid for such a long cultivation (Rengel and Jelaska 1986b).

If the conversion of NE to E cells really occurs in the culture of more mature cereal tissue, it means that non-embryogenic callus, induced directly on explant, represents true callus (»rapidly proliferating unorganized mass of dedifferentiated cells«) and not only the mass of proliferating organ primordia, which has been the common observation in cereal tissue culture (Cure and Mott 1978, King et al. 1978, Mott and Cure 1978, Wernicke et al. 1982). Accordingly, organogenesis in the cultures described here of more mature cereal explants is not »allowed« but »initiated«.

Stimuli for starting somatic embryogenesis

Whereas only induction of mitotic divisions in pre-determined E cells of immature explants is necessary for establishing E callus, redetermination of cells up to the embryogenic stage followed by repeated divisions is prerequisite for E callus induction from more mature explants. The question regarding stimuli for redetermination of cells has not been completely resolved yet.

Isolation of a particular cell from its neighbourhood, i. e. from the biochemical and physiological control existing in certain tissue, can make this cell develop as a separate unit (Williams and Maheswaran 1986). Severance or breaking of plasmodesmata caused by plasmolysis might be the necessary separation stimulus for cell redetermination and somatic embryogenesis (as was shown in the culture of carrot cells; Wetherell 1984). High osmotic pressure of media used for E callus induction in cereal culture was achieved either by increased percentage of sucrose added (Lu et al. 1982. 1983, 1984, Vasil et al. 1984) or by addition of L-proline (Armstrong and Green 1985, Rengel and Jelaska 1986b) or by addition of both L-proline and high percentage of sucrose (Kamo et al. 1985, Rapela 1985). The possibility that L-proline has exerted a specific positive effect on somatic embryogenesis may not be rejected. It should also be mentioned that a high percentage of sucrose was reported to have a detrimental effect on E callus formation in wheat culture (Ozias-Akins and Vasil 1982).

Separation of particular cells from the neighbouring ones can also be achieved by necrosis of surrounding cells which are inherent in increased susceptibility toward the factors which cause necrosis. Connection between somatic embryogenesis and necrosis of cells surrounding raising somatic embryoid was proposed earlier in grape (Krul and Worley 1977) and tobacco culture (Mii 1980). Here, it is not clear whether the separation of a certain cell from its neighbourhood by necrosed cells or, maybe, some metabolites synthetized during necrosis cause redetermination of a certain cell to the embryogenic stage. The necrosis causing factor connected with somatic embryogenesis in barley seedling tissue

culture was reported to be 6-benzylaminopurine (BA) (Rengel 1984, 1985, Rengel and Jelaska 1986a). In these experiments NE callus tissue necrosis was accompanied by browning of tissue which had been shown before to be influenced by various oxygenases (Lerch 1981). Biochemical reactions, catalysed by those enzymes, brought about the reduction of available oxygen which might induce somatic embryogenesis (as was shown in carrot cell suspension where a decrease in the level of dissolved oxygen, which led to an increase in the cellular level of ATP, was found to induce somatic embryogenesis; Kessell et al. 1977). On the other hand, BA by itself was reported to manifest positive effect on somatic embryogenesis in *Trifolium repens* (Maheswaran and Williams 1985) whereas additon of BA in the induction medium was also shown to induce necrosis of barley immature inflorescences tissue but, simultaneously, E callus induction in such cultures was inhibited (Thomas and Scott 1985).

Prolonged cultivation (6—8 weeks) without transfer onto the fresh medium may also be the factor involved in the induction of somatic embryogenesis (Rengel 1984, Weigel and Hughes 1985, Rengel and Jelaska 1986a, b), which was also observed in cotton cell culture before (Davidonis and Hamilton 1983). It means that decreased growth, caused both by exhausting the available nutrient supplies and by enhanced drying up of the callus tissue on the side opposite to the medium, can lead to somatic embryogenesis. Accordingly, certain stress conditions may be the necessary stimulus for somatic embryogenesis. Nevertheless, it is not clear whether the stress conditions would stimulate directly redetermination of cells up to the embryogenic stage or would only represent selection pressure in which the majority of cells die and only those with certain advantageous characteristics (and already redetermined) may survive, divide and form somatic embryoids. It should be mentioned that prolonged cultivation (longer than 3 weeks) was shown to decrease the formation of E callus and somatic embryoids in *Pennisetum purpureum* culture (Chandler and Vasil 1984).

An additional factor to which attention should be paid is the concentration of ethylene in the cultured tissue as well as in the gaseous environment. Verma and Tarka (1985) showed positive influence of 1-aminocyclopropane-l-carboxylic acid (immediate precursor of ethylene in higher plants) on somatic embryogenesis in carrot cell suspension. It should also be borne in mind that ethylene biosynthesis during senescence sharply increases, as was shown in the culture of rose cells shortly after the cessation of growth (LaRue and Gamborg 1971). This observation could be connected with delayed appearance of somatic embryoids in barley tissue culture (Rengel 1984, Weigel and Hughes 1985, Rengel and Jelaska 1986a, b) if the positive effect of ethylene on somatic embryogenesis in cereal culture might be demonstrated. In addition, Vasil (1983) stated deprivation of auxin being the most critical factor enabling development of somatic embryoids of cereals. Deprivation of auxin caused senescence in cultured pear cells (Balague et al. 1982) and increased ethylene production as well (Puschmann et al. 1985). Nevertheless, the possible connection between ethylene and somatic embryogenesis in cell and tissue culture remains to be established.

Beside the isolation of a particular cell from the surrounding tissue, the separation of certain tissue from another may be the stimulus for somatic embryogenesis. Botti and Vasil (1983) showed that the removal of mesocotyl tissue was the critical factor for E callus induction

from the apical dome of *Pennisetum americanum* mature embryo. On the contrary, only cultured complete barley mature embryo yielded E callus, whereas separate parts proliferated into NE callus only (R e n g e l 1987a, b).

In conclusion, it can be emphasized that physical isolation of a certain cell from its neighbourhood is likely to be one of critical factors enabling somatic embryogenesis. Escaping from the control of the tissue as a whole, enables a particular cell to develop on its own. The ways in which such separation can be achieved (breaking of plasmodesmata, necrosis of surrounding cells, stress conditions, senescence, etc.) depend on genetic and physiological factors as well as on culture conditions. It is not clear whether the difference in E callus induction, which exists among various tissues, depends on the different ability of cells of certain tissue to undergo both redetermination and divisions, or on the frequency of cells competent to respond in particular tissue. It also seems that more than one stimulus can separately start somatic embryogenesis in one particular tissue cultured *in vitro*. The developmental stage of explants is also the critical factor for E callus induction and only immature or very young (meristematic) tissues respond in the culture. Attempts to induce E callus formation from mature, fully differentiated cereal tissues remain to be achieved in the future.

There is an ultimate need for developing a suitable experimental system in cereal cell and tissue culture in which accurate biochemical and physiological measurements regarding somatic embryogenesis can be done. Improvement of our knowledge about somatic embryogenesis in cereal culture can make possible the use of this technique in breeding programs for these important crop species.

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SAŽETAK

ČINIOCI KOJI UVJETUJU SOMATSKU EMBRIOGENEZU U KULTURI TKIVA ŽITARICA

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Novi literaturni podaci o somatskoj embriogenezi u kulturi tkiva žitarica kritički su raspravljeni. Uspoređeni su rezultati postignuti u kulturi nezrelih eksplantata (embrio i cvatovi) s onima objavljenim za kulture zrelih embrija i dijelova mladih klijanaca. U kulturi nezrelih eksplantata izvor su somatskih embrioida stanice eksplantata, koje su bile embriogene i prije stavljanja u uvjete *in vitro,* dok je u kulturi zrelih embrija i dijelova klijanaca prvo potrebno postići određeni stupanj redeterminacije stanica. koje će onda biti izvor embriogenog kalusa. Čini se da embriogene stanice u kulturi zrelih embrija i dijelova klijanaca ne potječu izravno od stanica eksplantata, nego prvo nastaje neembriogeni kalus, čije stanice u određenim okolnostima prelaze u embriogene koje su izvor embriogenog kalusa i somatskih embrioida.

Jedan je od važnih uvjeta za indukciju somatske embriogeneze fizička izolacija određenih stanica od susjednih. Opisani su različiti činioci koji omogućuju takvu izolaciju, tj. izmicanje kontroli, koja postoji u tkivu kao cjelini (pucanje plazmodezmija, nekroza okolnih stanica. stresni uvjeti i starenje).

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