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Genetic stability / variability of regenerants obtained in culture of cells and tissues, its causes and mechanisms – A Review

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Abstract

The article presents an analysis of literature sources, that affect the issues of genetic stability/ variability of regenerants, obtained in cell and tissue culture. The reasons and factors due to which regenerants do not always retain the original genotype are considered. It is shown, that genetically stable material can be obtained with almost any method of regeneration under strict control of morphogenesis, occuring in cell and tissue culture, using histological, cariological and cytogenetic analysis of regenerating material. However, a higher percentage of the yield of genetically stable regenerants can be obtained using the axillary meristem activation method, direct somatic embryogenesis and formation of shoots directly from explant tissue, by passing the stage of callus formation on a nutrient medium.

Keywords: regenerants, genetic stability/ variability, cell and tissue cultures.

Introduction

Regenerants obtained through callus culture, as a rule, raise doubts about their genetic stability. In most cases, it is considered that this material is genetically unstable. This is confirmed by numerous cytogenetic studies, concerning regenerated plants, obtained in callus culture (Orbovic et al., 2008; Mohanty et al., 2008; Sivanesan, 2007; Nagai et al., 1984; De Buyser et al., 1988;Wang et al., 1989; Jha, 1989;Zinmy and Lorz, 1989;Sinska, 1988;Vapper and Kallak, 1986;Kovaleva and Dunaeva, 1987;Nehra et al., 1992;Popescu et al., 1997; Bulavin et al., 2021; Gajdošová et al., 2006; Galán-Ávila et al., 2020; Mazri et al., 2011; Navrotska 2017;).

There are many different reasons, due to which the plants, propagated in cell culture and tissues, do not always preserve the original genotype.

These causes can be of a dual nature: on the one hand, epi-genetic, causing short-term changes in the development of plant and not affecting its genome, on the other hand, genetic, affecting the cell nucleus.

Epigenetic changes

In the case of epigenetic changes, we can observe increased branching, changes in the arrangement of leaves, their shape, etc. However, these disorders are not associated with karyotypic changes, they are morphoses, that can be avoided by using appropriate concentrations of hormones and preserving sufficiently high reproduction rates. As a result of study of morphological disorders in regenerants of barley, expressed in opposite arrangement of leaves, Isaeva and Borodko (1988) on the basis of cytological analysis concluded, that the opposite leave arrangement is not associated with chromosomal disorders. They believe, that the appearance of plants with the opposite arrangement of leaves is due to the epigenetic phytohormonal disbalance of callus tissue caused by the presence in the nutrient medium of dichlorophenoxy acetic acid (further 2,4-D).

Based on the results, obtained from a thorough analysis of sugar cane regenerants, induced from leaf explants, Irvine (1984) concluded, that the presence of phenotypic changes is temporary and is the result of inadequate conditions of cultivation. Trofimets et al. (1979) arrived to a similar conclusion in a result of the study of potato regenerants, obtained in callus culture.

However, morphological deviations can also be of a genetic nature. In this connection, it is very important to determine whether these deviations have phenotypic character, not affecting the genetic nature of the plant, or they are caused by deviations in the genome.

Genetic disorders

The cause of abnormalities in the genome can serve characteristic feature of callus tissue, consisting in the presence of heterogeneous cells in it. The morphological heterogeneity of callus tissue can be associated with the type of explant, the composition of the nutrient medium, the conditions of cultivation, as well as the phase of growth of culture, the number of passages.

Besides morphological heterogenecity callus tissue has genetic heterogenecity. For such tissue, cytogenetic instability is typical, based on the content of cells with different ploidy, the formation of which is facilitated by many factors. This may be the effect of components of nutrient medium, the influence of metabolic products, the heterogeneity of the source material, the abnormality of correlative links in the allocation of the primary explant from the plant, the duration of the cultivation of plant tissues and cells.

The dominance of polyploid cells in callus culture may be due to the presence of auxins and cytokinins in the medium. This is confirmed by the studies of Torrey (1961), which testify to the role of kinetin in the induction of division of mainly polyploid cells of a heterogeneous explant, which leads to their dominance in comparison with diploid cells. 2,4-D and naphthylacetic acid (NAA) effects a similar influence on polyploid cells of callus. It is interesting to note, that in the callus, even if it was obtained from a non-polysomatic explant, polyploid and aneuploid cells usually begin to actively develop, especially when using 2,4-D or NAA (Sunderland, 1977).

It is known, that gymnosperms and many angiosperm plants, especially belonging to the families Apiaceae Lindl. And Asteraceae Bercht. and J. Presl, tissues and cells are differed in their diploid state, in other words, they are at different levels of ploidy. Such types of plants are considered to be polysomatic. Non-polysomatic species are those plants in which somatic reduplication is not observed during differentiation and therefore all cells of the organism are at the same level of ploidy. Species that are characterized by histological differentiation in the diploid state (non-polysomatic species) are few in number among angiosperms and constitute about 20% of all studied species (Sunderland, 1973).

It should be said that cells of the polysomatic explant are exposed to chromosomal endoreduplication. The degree of endoreduplication depends on the type of tissue. Thus, the meristematic cells of the differentiated parts of the plant (pericycle, procambium, cambium) remain diploid. A mixture of cells with different ploid levels (polysomatic or mixoploid) can be determined using the induction method of abnormal cell division (D'Amato, 1977).

Except to polysomatics, characteristic for angiosperm and gymnosperm plants, it is necessary to note the rather rare phenomen of mosaic of numbers of chromosomes in the apical meristems of some plants (the simultaneous presence of different aneuploid numbers of chromosomes and euploid cells). This type of mosaic, as and polysomatics, is a consequence of the genetic instability of the regenerants (Heinz et al., 1969; Vaarama, 1949).

Explant from non-polysomatic species of plants is euploid (all cells are at the same level of ploidy). When the explant has its origin from polysomatic species, the primary mitotic activity will be affect both diploid and endoreduplicate cells (D'Amato, 1977; D'Amato, 1964).

Thus, from the moment of cultivation, an explant can contain a heterogeneous population of cells, that reflects its state before being introduced into culture in vitro (for example, diploid and polyploid cells in polysomatic species) and is the result of the processes, occurring during induction of callus (chromosomal endoreduplication, mitosis, following nuclear fragmentation). During the growth of callus culture, there are enough opportunities for further change in the karyological apparatus, which depends not only on the type of explant, but also on external and internal factors.

As for polyploidy in the form of endoreduplication cells (endopolyploidy), it is already present in the explants of polysomatic species, and in the explants of non-polysomatic species is absent. One of the mechanisms of polyploidization of cells in an in vitro culture is the spindle break or delay of chromosome anaphase (D'Amato, 1977; Sunderland,1973).

Another mechanism of polyploidization in vitro is the nuclear fusion, binuclear or polynuclear cells (Sunderland,1973).

Consequently, nuclear fusion and cleavage of genomes during multinuclear anaphases can be a consequence of the formation of unpaired (triploid, five-ploid) numbers of chromosomes and their numerical predominance among dividing cells in callus culture. For cells of callus culture, aneuploidy is characteristic (the number of chromosomes that is not a multiple of 2n). A possible mechanism of the formation of aneuploid chromosomes is anaphase delay, multipolar spindles, bicentric chromosomes (D'Amato, 1977; Nandi et al., 1977; Sing and Harvey, 1975). Among the factors, that influence on this process, the most significant are the age of the culture and the composition of the nutrient medium (D'Amato, 1977; Nandi et al.,1977; Sing and Harvey, 1975;D'Amato, 1975).

Cytogenetic analysis of callus cultures

In some plant species, despite the occurrence in the culture of various ploid levels, plants with a diploid set of chromes are usually regenerated. Thus, the cytogenetic analysis of embryogenic callus cultures and plants-regenerants by *Papaver somniferum* L. showed, that with an increase in the age of callus, the number of cells with aneuploid and polyploid numbers of chromosomes is increased. However, all plants-regenerants had a diploid set of chromosomes (Wakhlu and Bajwa, 1987).

Gaponenko et al. (1987) conducted a cytogenetic analysis of callus cultures of barley, obtained from immature embryos, as well as plants-regenerants, and came to the conclusion, that regenerated plants have a high degree of heterogeneity of callus cultures and a diploid set of chromosomes.

Karyotypic variability was found in callus cells of garlic (Maggioni et al., 1989). According to the

authors, diploid cells dominated at the beginning of cultivation and accounted for 52%; by the fourth cycle of subcultivation, their frequency was decreased to 16%. Of the 92 plants-regenerants, 89 were diploid, 3 - tetraploid. Mohanty and Ghosh (1988) induced somatic embryogenesis and plant regeneration from leaf callus of barley. They carried out a karyological analysis of regenerated plants, indicating a normal diploid set of chromosomes (2n = 14).

A detailed cytogenetic study of cultures of callus cells and regenerated plants of *Cipura paludosa* Aubl. was conducted by Sengypta and Sumitra (1988). During cytological examination of calluses at the age of 40 weeks, the authors observed a high frequency of diploid mitosis, as well as the presence of hypodiploid and hyperdiploid cells. Plants, regenerated in tissue culture had a relatively stable karyotype. About 80% of the plants were diploids and had an initial parental karyotype. The remaining 20% of the plants had mosaic chromosomes. Wakhluand Bajwa (1986) regenerated homogeneous pepper plants by the way of indirect somatic embryogenesis, i.e. through callus culture. Cytogenetic studies of regenerated plants gave the basis for asserting about the genetic stability of the material, since the plants had a diploid set of chromosomes, and by the nature of meiosis, pollen fertility and morphological features were similar to plants, grown from seeds.

Genetically stable material was obtained by Zhou et al. (1988) from the callus culture of *Setaria italica* (L.) P. Beauv. The number of chromosomes in the cells of the roots of regenerants was 2n = 36, which was identical for cells of donor plant cells.

Radojevic (1988) regenerated plants *Aesculus hippocastanum* L. through indirect somatic embryogenesis. Plants regenerants had a diploid number of chromosomes (2n = 40).

The fact, that for some species of plants is possible to obtain genetically stable material from heterogeneous cells of callus culture suggests, that this process depends on the plant genotype. This can be confirmed by the regeneration of genetically stable shoots from callus of dracaena (Debergh, 1976), palm trees (Tisserat and DeMason,1980), coffee (Staritsky, 1970), amaryllis (Bapat and Narayanaswamy, 1976), peas (Yezhova et al., 1988) and other both woody and herbaceous plants.

The list of works, related to obtaining of genetically stable regenerants from callus culture could be continued. However, it makes sense to stop at the publications, which contain material about the genetic instability of the regenerants, obtained from callus tissue.

So, plants of wild diploid species *Solanum brevidens* Fil., obtained by Osifo et al. (1989) in the callus culture from explants of cotyledons and leaves were very diverse in their morphology and genetic structure. The authors state that about 70% of the plants that regenerated from the cotyledons of the callus tissue, and 20% of the plants that regenerated from the leaf callus tissue, were tetraploid. In the resulting regenerants, a correlation was observed between the morphological changes and the ploidy level.

A detailed cytological study of callus and sunflower plants regenerated from it was described by Cavallini and Lupi (1987). The authors claim, that counting the number of chromosomes in callus cells at the earliest stages showed a very low percentage of diploid cells - 11.2%. An interesting fact is that at the beginning of the development of shoots, the frequency of diploid cells in them reached only 21.2%, and the number of chromosomes in aneuploid cells varied from 17 to 33%. However, the frequency of diploid cells with further development of the shoots increased, and during flowering diploid cells (59.3%) and cells with 31-32 chromosomes (28.6%) dominated. Consequently, with the development of plants in vitro, diploid selection, which is intensifyed during flowering, occurs.

In the culture of cells and tissues of tomato haploids and diploids, Coornneef et al. (1989), by the way of cytological studies established, that plants, regenerated from leaf explants of diploids

were mainly diploids. Plants, regenerated from callus tissue and protoplasts, were mostly tetraploids. This is evidence of chromosomal instability in callus culture of cells of tomato.

In some cases, genetic variability was observed in plants regenerated from “mixed cultures”, which indicates the simultaneous induction of somatic embryogenesis and adventitious buds of shoots (Maddock et al., 1983; Earle and Gracen, 1985). According to the reasoning of Maddock et al. (1983), genetic variability may be associated with abnormal mitosis that occurs in large vacuolated cells during unorganized growth of callus, which is not observed in organs and meristems of culture in mitotically active cells or clusters of cells during organized growth. In this regard, the transition from a cytologically normal meristematic state, which is characteristic of embryogenic cells, to a state of differentiation leads not only to a change in chromosomes, but also to a loss of regenerative capacity.

Cells, originating from meristems, undergo cytological changes during differentiation, which leads mainly to the formation of tissues with homogenous ploidy. It is logical to assume, that the cytological organization of embryogenic cells and their ability to fast division possess the genetic stability inherent in meristematic cells. If this is so, then cultures of somatic embryogenic cells are generally genetically homogeneous, with the exception of some cells with modified genetic characteristics. Confirmation of our judgments can serve the results of numerous genetic studies of regenerants, received from callus embryogenic cells.

In the study of plants, regenerated as a result of somatic embryogenesis of cereals, according to Vasil et al. (1982), no polyploids and aneuploids were found in no species of the studied plants. The mitotic number of chromosomes, their meiotic configuration, pollen fertility, and the phenotypic and morphological characteristics of plants were similar to those of control samples. Somatic embryos, originating from cells with macroscopic chromosomal aberration, never return to their original state, since they do not develop beyond the early stage, do not reach maturity and therefore do not give rise to regenerant plants. However, on the basis of these studies one should not conclude, that genetic changes do not occur in somatic embryogenic cells — this would be a serious delusion.

In addition to the relative stability of material of cereals, obtained in the culture of cells and tissues, more exactly, regenerated by the method of indirect somatic embryogenesis, there is information in the literature about the variability of cereal regenerants (Orton, 1980;Cooper et al.,1986).

In the case of variability of material, changes are observed, on the one hand, in the number of chromosomes (polyploidy and aneuploidy), on the other - duplication of chromosomes, their inversion. Deeply convinced Larkin et al. (1984), in order to state the occurring genetic changes in regenerant plants, a thorough cytological study is necessary especially in meiotic cells, which is difficult to detect in species with a small number of chromosomes. Inherited, but recessive genetic changes can be detectedonly by studying of fertilized plants in the second and third-generations.

It should be noted, that in literature there is not so much convincing evidences of inherited genetic changes of regenerants, obtained in cell and tissue culture.

Thus, there is no doubt about the genetic stability of embryogenic callus, which gives rise to stable regenerants and arises from meristematic or undifferentiated tissue in young leaves, inflorescences, that originate from the meristematic tissue of the parent organism.

However, it is considered, that with long-term cultivation of callus cells, genetic changes occur in them, associated with the level of ploidy, structural rearrangement of chromosomes, accumulation of mutations, which leads to the loss of the former morphogenetic potential of cells.

This is confirmed by the studies of Bykova et al. (1988), in which it was shown, that in long-term passaged callus cultures of cotton, changes in the karyotype of the cells were observed: polyploidization, aneuploidization, mixoploidy. The authors come to the conclusion, that to use cotton callus tissues of cotton as model objects, we should take the tissues of the first passages, in the cells of which relative stability of number of chromosomes is observed, and for jobs that require a wide range of variability of number of chromosomes, is necessary to use tissues of later passages.

A detailed cytological analysis of callus cultures of *Scilla indica* Baker, conducted by Chakravarty and Sumitra (1987), showed, that with an increase in the time of cultivation of callus, the number of polyploid cells in it was increased. Calluses, which possessed by maximum regeneration ability, were in culture for about 120 days.

In the long-cultivated calluses *Crepis tectorum* L. (Sengupta et al.,1988), the karyotype underwent the following changes: initial explants, including roots and leaves, had cells with a normal diploid set of chromosomes (2n = 8); after one month of cultivation on an artificial nutrient medium, 95% of the callus cells contained a diploid set of chromosomes; calluses, cultivated on a nutrient medium, containing 2,4-D for one year had 62% diploid, 5% tetraploid, and 33% hyper-diploid cells. At the same time, the tops of the shoots of regenerants contained only cells with a normal diploid set of chromosomes (2n = 8). On the morphology the chromosomes of the regenerants did not differ from the source material.

It follows from the above, that in the population of callus cells of *Crepis tectorum*, which is heterogeneous in the number of chromosomes, the ability to form regenerants is associated with cells with a normal karyotype.

Recently, however, information has appeared in the literature concerning the stability of the karyotype in long-cultivated cells of callus and preservation by them of the morphogenetic potential.

A detailed cytogenetic analysis of long-term cultivated calluse and regenerants of pea obtained from them, was carried out by Yezhova et al. (1988).As a result of the studies, it turned out that long-cultured morphogenic calluses of pea contained mostly diploid cells (85%), although tetraploid, aneuploid and cells with chromosomal aberrations were found, that could be involved in the morphogenesis process. It is interesting to note the fact, that according to the authors of these results, in the case of seed reproduction of regenerants, genotypes with a diploid set of chromosomes were selected, which contributed to the elimination of most material with karyotypic changes. This once again confirms the idea, that in heterogeneous in the number of chromosomes population of callus cells, this time pea, the ability to form genetically stable regenerants is associated with cells having a normal karyotype.

Cells of the embryogenic callus of Javanese citronella (a medicinal plant) were passaged for 2 years (Sreenath and Jagadishchandra, 1989). As a result, they preserved the original set of chromosomes (2n = 20). Regeneranted plants had a morphology, similar to donor plants, as well as the same number of chromosomes.

Chen et al. (1987) studied the regeneration potential of the explants of the apex of the shoot, stem, leaf, cotyledon, and the roots of two varieties of papaya. According to the authors, the high regenerative capacity of callus cultures has been persisted for 2 years. Normal plants were developed from somatic embryos.

According to studies by Jelaska et al. (1985), the selected lines of callus of *Cucurbita pepo* L. have not lost their ability to embryogenesis even after 3 years. Similar results were obtained by Gresshoff (1980) in the study of *Trifolium repens* L. and Fassuliotis et al. (1981) in the study of *Solanum melongena* L. Successful regeneration of plants was achieved from long-term cultivated callus in such plant species as: *Medicago sativa* L. (Stavarek et al., 1980), *Pisum sativum* L. (Hussey and Gunn, 1984), *Stylosanthes guyanensis* (Aubl.) Sw. (Meijer, 1984), *Lotus corniculatus* L. (Orshinsky and Tomes, 1985).

From our point of view, such an ambiguous reaction of callus cultures to long-term cultivation can be explained by the species affiliation of the material, the type of explant, its physiological state, the composition of the nutrient medium, the ratio of growth regulators, the conditions of cultivation and a number of other unaccounted factors.

Returning to the question of the genetic variability of regenerated plants, obtained in callus culture, it should be said, that the genetic variability of the material can be used as a source of useful forms in plant breeding (Evans, 1987,1989; Evansand Sharp, 1983;Lorz et al., 1988). For example, cytological studies of Boladjiev and Cuong (1988) of some rice cultivars and hybrids F1, obtained in callus culture, showed, that there were 28% of haploids among regenerants, 67% of diploids, 4% had a set of chromosomes above the diploid level. The study of the content of pigments and photosynthetic activity in the hybrid F1 showed, that diploids of this hybrid are of practical value for selection.

Genetic analysis of the seed progeny of variable regenerants shows, that in many cases the cause of variability is mutations of single or small groups of genes of dominant, semi-dominant or recessive character in the nuclear, plastid or mitochondrial genomes, as well as the high frequency of chromosomal rearrangements (Lörz et al.,1988).

Some variability, traced in the tissue culture is a continuation of the existing mixoploidy and the heterogeneous nature of the explant. It can occur as a result of the action of the components of the nutrient medium, the loss of spatial and temporal control of cell division and differentiation, which is also characteristic of intact tissue of plants. It seems, that the variability, observed in plants, regenerated from differentiated tissue of the core of the stem or mesophyll of the leaves, is similar to natural variability.

Genetic variability may be associated with the initial variability of somatic cells of the explant or occur in the process of artificial cultivation of cells and tissues. These and other questions concerning the karyological changes of regenerated plants, changes in the genome at the molecular level, changes, affecting the nucleus and organelles are described in detail in the review by Lörz and Brown (1986).

It should be noted, that during the reproduction of valuable genotypes and their long-term storage in vitro, when it is necessary to obtain the full identity of regenerated clones, genetic variability is undesirable in this situation.

Conclusion

Unfortunately, in the literature there is still no clear differentiation of views on the question of which method of regeneration can be used to obtain genetically stable material, and for which one - variable. Probably, at this stage of knowledges, accumulated on the genetic stability / variability of regenerants, there are no valid arguments in favor of one or another method. And this is not accidental, since the genetic stability / variability of the material, obtained in the culture of cells and tissues depends not only on the method of regeneration, but also on other numerous factors that were discussed in this article.

Despite the complexity of the problem, regarding the quality of regenerants, obtained in culture of cells and tissues, analysis of the literature leads to the conclusion, that genetically stable material can be obtained with almost any method of regeneration under strict control of morphogenesis, occurring in culture of cells culture and tissues, using histological, karyological and cytogenetic analyzes of the regenerated material.

However, the highest percentage of yield of genetically stable regenerants can be obtained by using the method of activation of axillary meristems, direct somatic embryogenesis and formation of sprouts directly from the tissue of explant, by passing the stage of callus formation on the nutrient medium.

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