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THE MORPHOLOGY OF ABNORMAL EMBRYOIDS AND PLANTLETS OBTAINED FROM EMBRYOGENIC CALLUS OF PUMPKIN (*CUCURBITA PEPO* L.)

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Introduction

Numerous embryoids and plantlets with various morphological abnormalities have been found in the strains of embryogenic callus of pumpkin (*Cucurbita pepo* L.) (Jelaska 1974). Similar phenomena have already been observed and described in several other plant species (Nakajima and Yamaguchi 1967, Takeuchi 1968, Vermylen-Guillaume 1969, Konar, Thomas and Street 1972 and others) as well as in excised embryos cultured on synthetic media (Sanders 1950, Rijven 1952) or in embryos after in vivo treatment with growth regulating substances (Haccius 1955; Haccius et al. 1960, 1965).

In this paper only morphological aspects of abnormal ambryoids and plantlets are presented, i. e. without any anatomical or histological data. In the following text the term embryoid is used to describe embryo-like formations and the term plantlets to characterize seedling-like structures. These two terms thus refer only to the morphological aspect, regardless of the possibly different ways of development.

Abbreviations

2,4-D = 2,4-dichlorophenoxyacetic acid
IAA = β-indolylacetic acid
IBA = β-indolylbutyric acid
NAA = α-naphtylacetic acid
y. e. = yeast extract
MSC = complete medium after Murashige and Skoog (i. e. containing salts and organic components)
MSS = medium after Murashige and Skoog containing only salts (without organic components)

Material and Methods

The methods of culturing the pumpkin embryogenic callus and the conditions under which these cultures reveal their morphogenetic abilities have been described previously (Jelaska 1972, 1974).

The tissues were grown on a complete medium after Murashige and Skoog (1962) with the addition of 30 $g.l^{-1}$ glucose, 9 $g.l^{-1}$ Difco Bacto agar and one of the following growth substances: IAA, IBA, NAA, 2,4-D and y. e. Occasionally tissues were transferred from a medium containing one type of growth substance to a medium with another growth substance, as shown in Figs.

The cultures were kept at 26 ± 1 °C under artificial light (fluorescent tubes, 40 W, 220 V, 6500 °K) for 16 hours light and 8 hours darkness daily at an illuminating intensity of about 500 lx.

For the microscopic investigation of outer morphology the embryoids, plantlets and cell aggregates were fixed for 24 h in ethanol acetic acid mixture (absolute ethanol : glacial acetic acid — 3 : 1). The fixed material was examined under a Zeiss-Jena dissecting microscope and drawn by a Zeiss-Jena camera lucida.

The line drawings show characteristic stage of anomalies which were found in various passages and on various media (i. e. on media with various growth substances).

Results

Strains of embryogenic pumpkin callus were grown on agar medium and were transferred to fresh media every 6—8 weeks. The investigated embryoids and plantlets were taken from cultures of the same age i. e. 6 weeks old. These cultures were characterized by a great number of embryoids and plantlets showing a wide range of deviations from normal ontogenetic models. Each of the following figures shows embryoids developed in the same test tube.

a) *Abnormal development of the cotyledonary portion*

Great abnormalities in the development of the cotyledo were observed. Heterocotyly (Figs. 3C; 6B, F; 8b), monocotyly (Fig. 8d), tricotyly (Figs. 1D; 6F; 7B, C, E; 8f), quadricotyly (Figs. 4B; 8e), syncotyly (Figs. 1J; 2A, D; 4C; 7D; 8a) and leaf-like cotyledons (Figs. 1A, E; 4B; 8b, d) were noticed.

Anomalies of this kind appeared in the cultures regardless of the growth substance in the culture medium (2,4-D, IBA, IAA or NAA) and even when the callus was transferred to a medium containing no growth substance, embryoids with various anomalies mentioned above appeared as a rule. Leaf-like structures appeared in particularly large numbers in cultures grown on media containing IBA and measured 5—10 mm (in length) or even more.

b) *Root formation*

In pumpkin callus the development of rootlets could also be found. On cell clumps resembling rather abnormal embryoids only the rootlet was well developed (Figs. 1F; 2B; 4A, G, I; 5G). These forms were also found regardless of whether the tissue grew on 2,4-D, IBA or NAA.

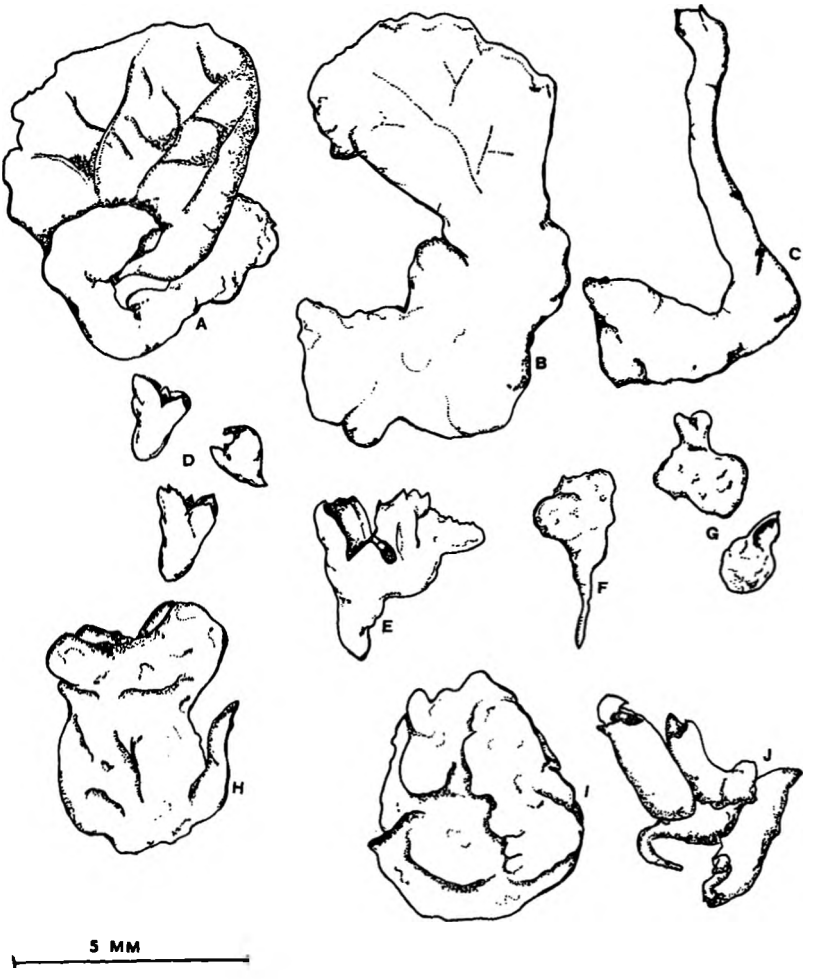

Fig. 1 A—J. Abnormal growth of pumpkin embryoids in vitro. Embryogenic callus (clon Pea) was cultured on MSC + 1 mg.l^{-1} IBA, 2nd passage.
A and B big leaf-like structures; C plantlet with elongation of the hypocotyl and poor in the development of cotyledonary portion; D tricotyleous embryoids; E embryoid with leaf-like cotyledons and reduced root; F undifferentiated mass with root-like structure; G undifferentiated masses with leaf-like structures; H and I undifferentiated masses like an abnormal embryo; J syncotyleous embryoids.

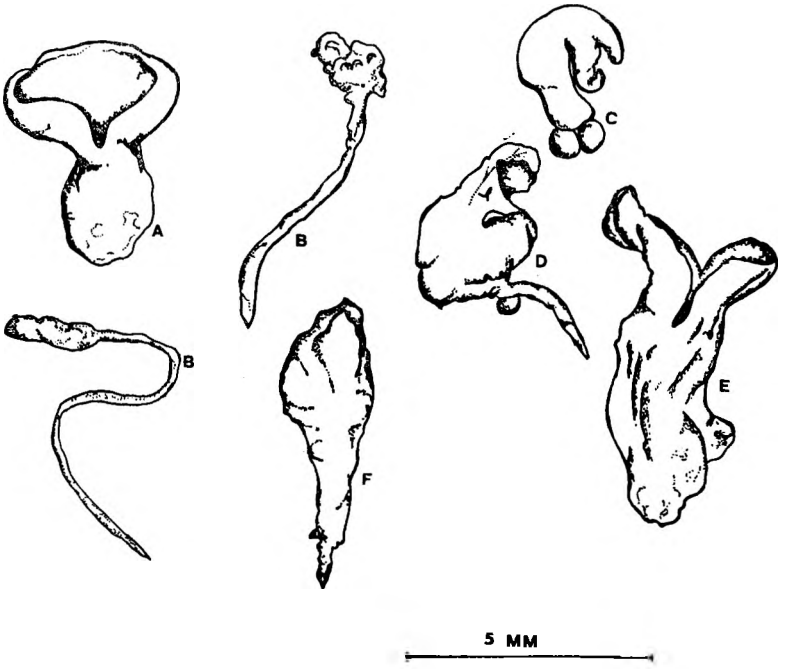

Fig. 2 A—E. Abnormal growth of pumpkin embryoids in vitro. Embryogenic callus (clon $Ž_5$) was cultured on MSC + 1 mg.l^{-1} IBA, 18th passage.
A syncotyleous embryoid without root; B undifferentiated cell masses with well developed roots; C embryoid with undeveloped root and two globular cell masses (adventive proembryoids?); D syncotyleous embryoid with well developed root and abnormal hypocotyl portion; E undifferentiated embryoid with poor development of hypocotyl and root portion; F undifferentiated embryoid on cotyledonary portion.

Fig. 3 A—D. Abnormal growth of pumpkin embryoids in vitro. Embryogenic callus (clon MSS) cultured on MSS + 1 mg.l^{-1} IBA, 11th passage.
A and B undifferentiated cell masses with leaf-like structures; C embryoid-like structure with abnormal heterocotily; D embryoid-like undifferentiated mass.

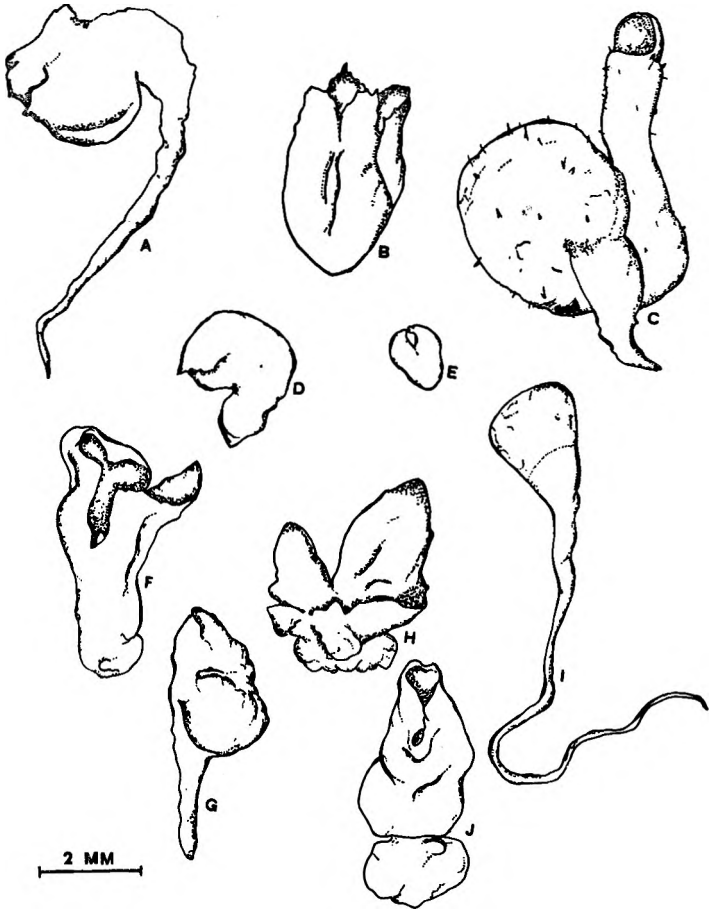

Fig. 4 A—J. Abnormal growth of pumpkin embryoids in vitro. Embryogenic callus (clon Wc) was cultured on MSC + 0.3 mg.l^{-1} 2.4-D + 2 g.l^{-1} y. e., 14th passage.
A, G and I embryoid-like structures with well developed roots; B quadricotily combined with syncotily; C syncotyleous embryoid with giant growth of hypocotyl portion; D and E embryoids with normal appearance; F and J embryoids with poor development of root's portion and abnormal development of cotyledons; H leaf-like structure.

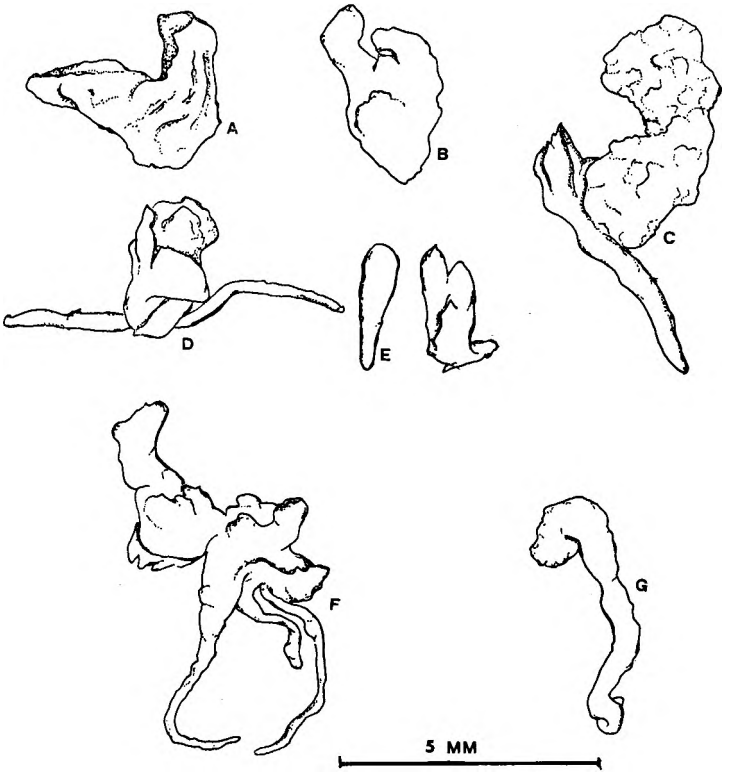

Fig. 5 A—G. Abnormal growth of pumpkin embryoids in vitro. Embryogenic callus (clon ANA) was cultured on MSC + 1 mg.l^{-1} NAA + 13.5 mg.l^{-1} adenine, 14th passage.
A and B undifferentiated masses like an embryo; C plantlet with undifferentiated cell mass on one part of its surface; D abnormal twin; E club-like embryoids; F multiple fasciated growth; G embryoid-like structure with poor development of cotyledonary portion.

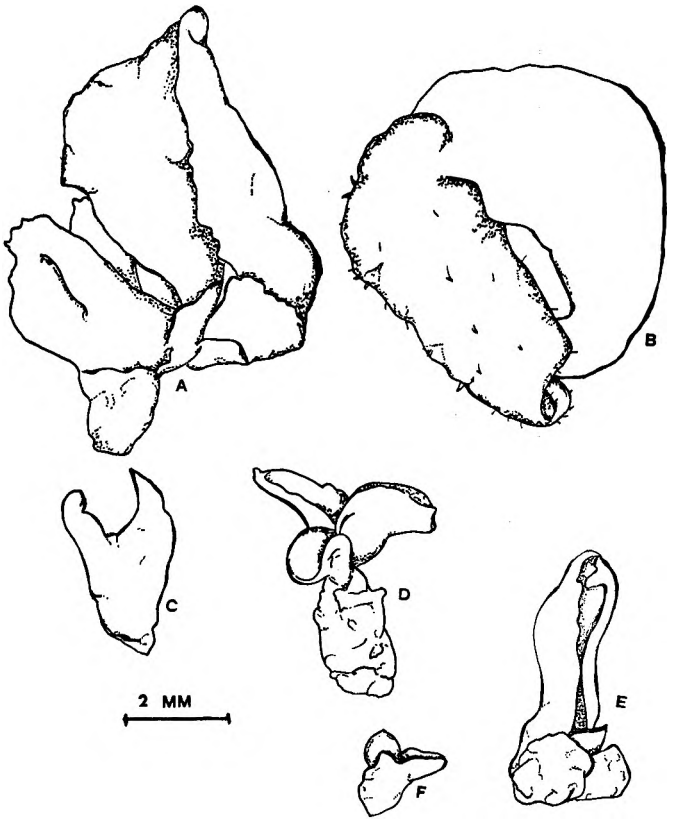

Fig. 6 A—F. Abnormal growth of pumpkin embryoids in vitro. Embryogenic callus (clon Wc) was transferred on MSC + 1 mg.l^{-1} IBA in 11th passage. Previously it was cultured on MSC + 0.3 mg.l^{-1} 2,4-D + 2 g.l^{-1} y. e.
A and E leaf-like structure; B giant heterocotyleous embryoid with hairs on its surfaces; C normal embryoid; D bud; F tri- and heterocotyleous embryoid.

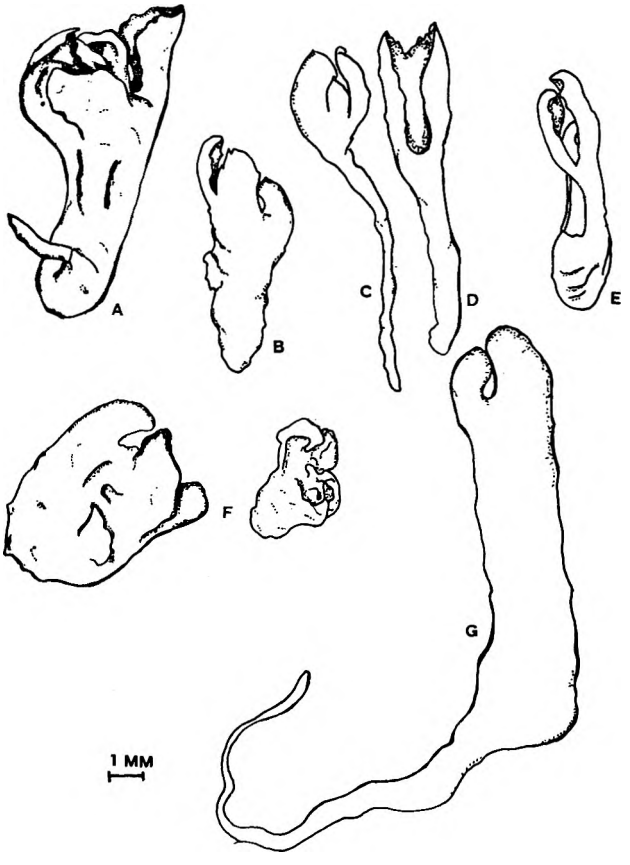

Fig. 7 A—G. Abnormal growth of pumpkin embryoids in vitro. Embryogenic callus (clon Z_3) was cultured in 4th passage on MSC + 0.5 g.l^{-1} glucose + 0.1 mg.l^{-1} IAA. Previously it was cultured on MSC + 3 g.l^{-1} glucose and 1 mg.l^{-1} IBA.
A polycotyleous embryoids; B, C and E tricotyleous embryoids with various abnormal growth of hypocotylary portion; D syncotyleous embryoid; F undifferentiated cell mass like an embryoid; G giant embryoid with broad hypocotyl and small cotyledons.

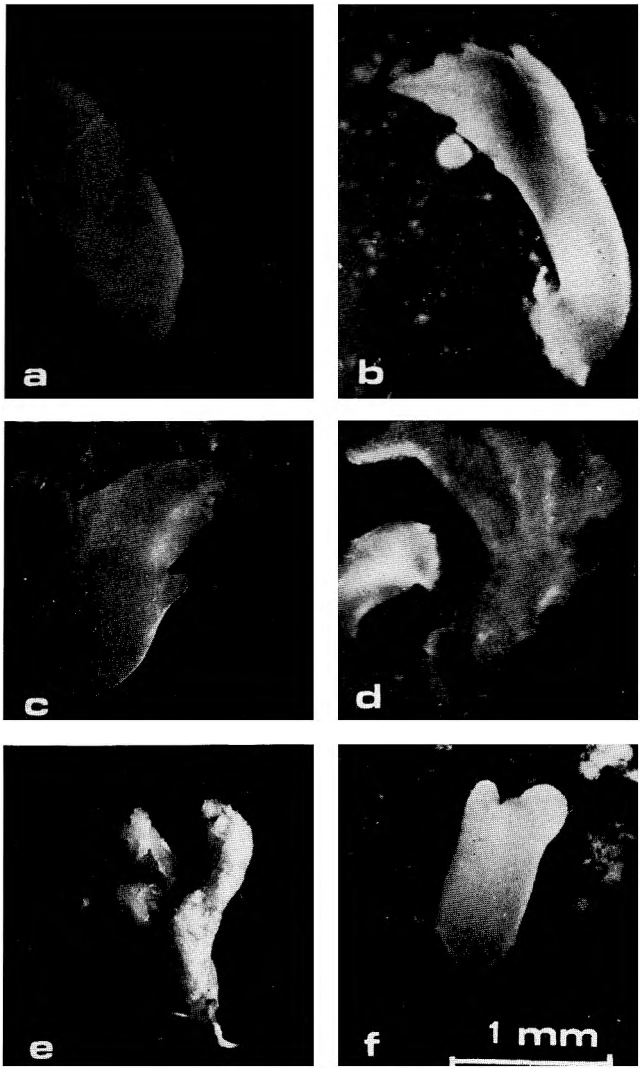

Fig. 8 a—f. Photographs of some abnormal pumpkin embryoids. a — syncotyleous embryoid; b — heterocotyleous embryoid with leaf-like cotyledon; c — lobed embryoid; d — monocotyleous embryoid with leaf-like cotyledon; e — quadricotyleous plantlet with callus on surface; f — tricotyleous embryoid with poor development of cotyledonary portion.

c) *Undifferentiated cell masses*

Cell masses frequently appeared in cultures and — although of an undiferentiated appearance — they resembled abnormal forms of embryos (Figs. 1H, I; 2F; 3A, D; 5A, B; 7F).

The surface of several embryoids and plants was frequently coverd with callus or the embryoid developed on one side of the callus mass (Figs. 5C, D; 8c).

d) *Fasciated growth and twins*

These two abnormal types of growth also occurred in pumpkin callus (Figs. 5D, F). The development of the hypocotylary portion was very often abnormal (Figs. 1B; 2E; 7A, C, D, G).

e) *Size of embryoids and plantlets*

The size of embryoids and plantlets varied greatly and it was not possible to observe any regularity. Several embryoids of different sizes appeared in the same culture and gigantic forms were found together with dwarfs, both belonging to the same developmental stage, as e. g. in Figs. 4B and E.

Specific differences observed in the effects of the growth substances are the following: 1) The presence of IBA in the medium stimulated better development of cotyledons and leaves, while 2,4-D inhibited their growth. 2) The transfer of callus from the medium containing 2,4-D to e. g. a medium with IBA induced a better growth and development of embryoids in comparison with the earlier passage. 3) On the media with 2,4-D the embryoids were on an average smaller than those growing on the media with IBA or especially with IAA.

Discussion

All anomalies found in embryoids of pumpkin were observed — more or less — also in the development of embryos of other species cultivated in vitro, regardless of whether they were somatically induced embryoids or zygotic embryos. Attempts have been made to determine the causes of these numerous aberrations. Haccius (1955), Haccius and Trompeter (1960) and Haccius and Frey (1965) were able to show experimentally that certain growth substances (2,4-D, NAA and others) applied to *Eranthis* seeds, (containing a young undifferentiated embryo) cause in 100% of the cases an abnormal cotyledon development of the syncotyly or polycotyly type or twins (3—8%).

Rijven (1952) and Sanders (1950) experimented with young isolated embryos of *Capsella* and *Datura* cultivating them on different media. Anomalies, as described here for *Cucurbita pepo*, were observed also in their material. Johri and Sehgal (1966) achieved a development of abnormal embryos and polyembryony in ovaries of *Anethum*, *Foeniculum* and *Trachyspermum* by means of caseine hydrolysate, IAA and yeast extract. The authors belived them to be consequence of the added substances.

A range of such anomalies were found by Konar et al. (1972) in the culture of cell suspensions of *Atropa belladonna* L. Other investigators, studying the morphogenetic capacity of tissues in culture, also

observed such teratological phenomena, especially in carrot, but they did not analyse them in detail considering that the complex growth substances were responsible for this kind of development. More attention to development of this kind was paid by Vermylen-Guillaume (1969) in studies of callus cultures of carrot. It is interesting to note that under experimental conditions she obtained a development of abnormal embryoids even in the absence of growth substances. I noticed the same phenomenon in pumpkin callus when I transferred it to a medium without any growth substance added, although they are supposed to be responsible for this kind of development.

In the experiments of Vermylen-Guillaume neither the type nor the concentration of sugar affected the kind of abnormal growth.

Sanders (1950) obtained very similar abnormalities in culture when experimenting with *Datura* zygotic embryos which all originated from fertilized egg cell. They often developed in culture into undifferentiated cell masses. Similar comparisons with embryos and embryoids were carried out by Nakajima (1962) who could show that in tissue culture both were inclined to abnormal growth, especially in cotyledon development. Similar observations were made by Maheshwari et al. (1963) and Johri and Bajaj (1963).

All these data from literature support the conclusion that the origin of abnormal forms (of the embryoid during its growth and development) should be attributed to the influence of physical and chemical conditions of the medium rather than the effects of different developmental ways. Wardlaw (1965) says: "Tukey (1938) observed that by culturing excised immature embryos of fruit trees under certain conditions, the ontogenetic growth pattern could be modified in a characteristic manner: the embryos did not follow the usual path of development but grew into plantlets which exhibited a different but definite conformation. The normal ontogenetic development of the embryo is not, in fact, inevitable but is the necessary result of factor present in the environment... Mature, or nearly mature, embryos also exhibited unusual types of growth, but these were unlike those obtained from very young embryos... His finding reminds one of Goebel's view that organisms are constantly changing throughout their ontogenetic development and that genetical constitution alone does not determine morphogenesis and funcional activity... The failure of embryos to follow the "normal" pattern of embryonic development outside the embryo-sac raises the question as to the nature of the environment which brings about "normal" development."

Summary

In addition to normal forms of embryoids in an embryogenic callus of pumpkin, various abnormal forms also appear. They can be characterized, according to their morphology, as monocotyleous, heterocotyleous, tricotyleous, quadricotyleous, syncotyleous, and embryoids with leaf-like cotyledons.

Other forms observed include: twins, forms with undeveloped roots, undeveloped hypocotyls, callus formed on the surface of the plantlet, multiple growth and forms of undifferentiated masses. The anomalies described, with the exception of certain differences in the size of the embryoids are not dependent on the presence of a specific growth substances in the nutrient medium.

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SADRŽAJ

RAZVITAK ABNORMALNIH EMBRIOIDA U EMBRIOGENOM KALUSU BUNDEVE (*CUCURBITA PEPO* L.)

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Pored normalnih oblika embrioida stvaraju se u embriogenom kalusu bundeve i raznovrsni abnormalni oblici, koji se po svojoj morfologiji mogu okarakterizirati kao monokotilni, heterokotilni, trikotilni, kvadrikotilni, sinkotilni i embrioidi s lisnatim kotiledonima.

Osim embrioida s abnormalnim kotiledonima zapaženi su oblici: srasli embrioidi (blizanci), s nerazvijenim korijenom, s nerazvijenim hipokotilom, sa stvaranjem kalusa na površini biljčice, multiplim rastom i nediferenciranim rastom. Opisane anomalije, s izuzetkom izvjesnih razlika u veličini embrioida, neovisne su o prisutnosti specifične supstancije rasta u hranidbenoj podlozi.

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