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Microclonal propagation of some bulbous and cormous plants based on regeneration processes in morphological different explants

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Summary: Morphological processes in explants of leaves, axes and shoots of lilies, hyacinth, daffodil and gladiolus during regeneration *in vitro* undergo gemmorhizogenesis. Shoot formation precedes the differentiation of adventive roots. Practically all living tissues of explants including the parenchyma sheath of vascular bundles are involved in the formation of meristematic clumps. As a rule epidermis doesn't take part in this process immediately. It is due to its much earlier and deeper specialization. During regeneration, the compound branched hydrocyte system differentiates in explants of different morphological nature. It serves redistribution of inner resources within explants and resources of the culture medium as well which is necessary for the differentiation of *de novo* shoots and adventitious root rudiments. Differences in morphogenetic reactions *in vitro* of explants of different morphological nature are non-significant and they depend only to a less degree on its taxonomical affiliation.

Keywords: lily, hyacinth, daffodil, gladiolus, regeneration, morphogenesis *in vitro*, polyads, meristematic clumps, gemmorhizogenesis

Loss of biodiversity is one of the global ecological problems which has been raised even before mankind. Nowadays the scope and intensity of meddling with ecosystems have lead to essential degradation and fragmentation of natural areas and to a decrease of species quantity, which along with global climate changes result in a severe loss of biological diversity (Heywood & Iriondo 2003). Applications of new approaches and technologies of preserving biodiversity of genetic resources are essential for solving the problems of natural area destruction, restoring of species, etc. One of such new approaches is the application of biotechnologies which are developing quickly now and have considerable potential in future.

Microclonal propagation is a high effective technology of propagation of rare, genetically unique plant forms, valuable varieties of agricultural and ornamental cultivars. It allows to realize more completely the abilities for regeneration and it also has advantages compared to traditional methods of vegetative plant propagation.

Plant cell, tissue and organ culture is a convenient model system for studying the cytodifferentiation processes and development pathways because morphogenetic reactions respond to experimental influence of different factors. It can be applied with great success for a better understanding of many general biological problems. In this purpose, complete investigations with a wide spectrum of different contemporary methods of experimental botany and data from different spheres of biology as well are essential.

Microclonal propagation technology is based on cell, tissue and organ culture with a long history. The idea of cultivation of isolated plant parts was predicted in many proceedings in the 19th century.

Haberlandt (1902) was the first who published the idea of *in vitro* plant cell cultivation and he formulated the concept of cell culture. The first experiments with isolated plant cells on artificial

culture medium failed. The cells were alive during several months, but they didn't undergo divisions. The main reason of failure was the use of culture mediums with a relatively common mixture, which didn't satisfy the requirements for nutritive and growth stimulating substances of isolated cells, and to some extent the improper choice of object (highly specialized cells and tissues which have lost their meristematic activity). Inspite of this, the results of Haberlandt's experiments gave the considerable impetus to further investigations of processes which take place *in vitro*. He predicted the application of this method as wonderful facility to study different physiological and morphological problems (Haberlandt 1902).

White (1939) and Gautheret (1939) are rightly considered as founders of the method of isolated plant tissue cultivation. First significant investigations of different culture mediums and application of *in vitro* culture technique for studying tissue differentiation under the influence of different exogenous and endogenous factors were carried out by them, too. On the basis of these investigations further method improvements have been made. Not only the technique of tissue cultivation on the surface of solid agar culture mediums, but also the methods of growing of suspensious cultures and single isolated cell cultivation techniques were elaborated in detail. In 1958–1959 the somatic embryogenesis phenomenon in carrot tissue culture was discovered and described for the first time by Steward et al. (1958) and Reinert (1959). Cell selection and interspecies hybridization based on tissue culture methods began to develop. At present, cells and tissues of vegetative organs, isolated embryos and pollen grains of plants of different systematic groups are grown *in vitro*.

The very first experiments on *in vitro* culture were carried out mainly with herbaceous or woody members of different dicotyledonous families. Gautheret (1959) described tissue culture protocols for 100 plant species, but he mentioned only ten species of monocotyledons. Pretentious investigations of monocotyledonous species were undertaken in 1940s to 1950s (Loo 1945; Galston 1948; Morel & Wetmore 1951; etc.). The objects of investigations in the first instance were bulbous and cormous plants with high ornamental qualities. Some authors mentioned the dependence of growth intensity and shoot differentiation on quantative and qualitative content of nutritive substances and hormonal growth regulators (Robb 1957; Niimi 1986), as well as cultivation temperature, light spectrum and intensity (Matsuo 1975; Stimart & Asher 1981). The regeneration ability were studied by means of different explants: buds, nodal parts of corm, bulb scales, flower stalk segments, basal parts of young leaves, twinned scales with a part of corm, etc. Hackett (1969), Ivanova & Kozitsky (1981) and Mitrofanova & Ivanova (1987) studied the interconnection between the intensity of morphogenetic processes, in particular, the quantity of developed bulbils and orientation of explants on culture medium.

The basis of *in vitro* culture method is totipotency: a unique feature of plant cells which provides reserves for polyvariation of plant reproduction in extreme conditions. However, it seems to be more proper to talk about the level or the degree of totipotency (Batygina 1984). The difference of morphogenetic ways of development in different plant species as well as the choice of the way of morphological structure formation depend on it. Probably the variation of cell and tissue reactions to different factors in dependence on the plant genotype is connected with this fact, too (Murashige & Huang 1985). Steward et al. (1958) noticed that any diploid cell is potentially totipotent. Later it was shown in *Nicotiana tabacum* (Zagorska et al. 1971) that morphogenetic capacities are characteristic not only of diploid cells but of polyploid cells as well. However, the degree of realization of this potency is due to the specific cytogenetic nature of the cell.

In connection with this fact it is necessary to identificate the initial cells *in vitro*, which give rise to different growth processes. Theoretically, all plant cells are able to regenerate the whole organism. Nevertheless the expression of this ability confined to meristematically competent cells which are able to react to changes in culture medium. Their derivates are able to differentiate into shoots, roots, etc. The availability of an inductor in the culture medium is insufficient for a specific morphogenetic pathway of development of the cell. The combination of its action with the readiness of the cell to react to it as well as some other factors are necessary (Butenko 1984). These initial cells differ from common parenchymal cells by some features. They have a large nucleus, increased nucleus-plasmatic correlation, dense basophilic cytoplasm, small vacuole with numerous cytoplasmic bundles. They often have much storage starch and sometimes lipids (Thorpe & Murashige 1970). In culture mediums with hormones the initial cells divide multiple (segmentation) and form a spheric mass of small isodiametric cells (Butenko 1984). The development of meristematic centers and induction of their morphogenesis are accompanied by changing of subcellular structure and metabolism (in particular, the intensity of cell respiration increases). Some authors noticed an increase of enzymes of glycolysis and pentose phosphate pathway during the induction of morphogenesis and the intensifying of protein and RNA synthesis as well (Danilina 1972; Mohamed & Dmitrieva 1974; Thorpe 1977). Due to increasing synthetic activity the cells of meristematic centers and cells of developing morphological structures attract more nutritive substances.

Different morphological processes *in vitro* need systematization and a revealing of definite regularities which are characteristic of them. Batygina (1978, 1991), Batygina et al. (1978) and Batygina & Vasylieva (1983, 2002) pay great attention to the research of varieties of morphogenetic processes *in vitro*, to their classification and elaboration of terminology as well. The authors concluded on the universality of morphogenetic pathways in nature and in experimental conditions (including *in vitro* conditions) and interpreted them as plant reproductive strategies. Thus, a new individual can develop be means of embryogenesis, embryoidogenesis and gemmorhizogenesis as well (Batygina & Vasylieva 2002). We consider this approach rationally.

Inspite of long history of isolated plant cell, tissue, organ and embryo culture methods *in vitro*, as well as abundance of biochemical, physiological data and some other aspects, characteristics of morphogenetic transformations from the very first stages have been insufficiently studied. Haccius contributed to the study of morphogenetic processes *in vivo* and *in vitro* significantly (Haccius 1965, 1973, 1978; Haccius & Bhandari 1975; Haccius & Lakshmanan 1969). Her papers deserve special attention. The origin of embryoids or somatic (non-zygotic) embryos, comparison of their first stages of development with zygotic embryos, attempts to find differentiation criteria, terminology and classification as well are discussed there.

The majority of articles on morphogenesis *in vitro* is devoted to physiological and biochemical aspects of this process. In particular, little attention has been paid to the differentiation of the vascular system. Meanwhile, studying the preliminary changes of cytoplasm from the very first stages (Sinnot & Bloch 1945) became possible by using plant cell, tissue and organ culture *in vitro* as a model. Among the investigations concerning this problem the paper of Gamaley (1972) on the cytological basis of xylem differentiation should be noticed. In particular, he examines the ultrastructure of tracheal elements and their metamorphosis during xylem differentiation. Unfortunately, considerably little attention has been paid to literature about sieve element differentiation.

As mentioned above, most investigations of morphogenesis *in vitro* were dedicated to dicotyledons and first of all to Liliaceae, the extraordinary interesting group from theoretical and practical viewpoint as well. Many of them are remarkable for their ornamental qualities and they are source of medical and food stuff, too.

To date relatively few investigations concern morphogenetic processes of *in vitro* cultures of bulbous and cormous plants. Reports of Naylor (1940) and Walker (1940) deserve attention. In the first article the formation and orientation of cell walls as well as some distinctive features of first divisions are noticed and initial stages of bulbil formation in leaf explants of hyacinth are described. Walker (1940) published interesting facts about initial stages of the formation of meristematic activity centers (adventitious bulbs differentiate later) in bulb scales and about the place of primordial initiation of roots in his work on the regeneration *in vitro* in bulb scales of lilies. He noticed the regenerative activity of epidermal cells. Yarvekulg (1965) presented rather fragmentary facts on regeneration of leaf cuttings of hyacinth and bluebell, on localization of first cell divisions and on formation of meristematic activity areas. In all mentioned articles a consequent morpho-anatomical description of developed new structures, their features and connection with tissues of explants as well as vascular element differentiation are absent.

In this paper the main attention is paid to the comparative analysis of morphogenesis from the very first divisions of initial cells to the formation of adventitious buds and roots in explants of different morphological nature (cataphyll, medial leaf, axis, bud) *in vitro*.

In our opinion, additional information on the regularities of morphogenesis during the regeneration *in vitro* of different plant species would contribute to the development of experimental morphology and practice of microclonal plant propagation.

Materials and methods

We studied *Lilium regale* Wils., *L. longiflorum* Thunb., *L. speciosum* Thunb., *L. pardalinum* Kellog., *L. candidum* L., *L. martagon* L. (Liliaceae); *Hyacinthus orientalis L.* cv. 'Anna Marie' (Asparagaceae); *Gladiolus hybridus* cv. 'Dixiland' (Iridaceae); *Narcissus hybridus* hort. cv. 'Geranium' (Amaryllidaceae). Selection of organs for explants and date of experiment were realized according to Hussey (1980) and Rumynin & Slyusarenko (1989). The preparation of culture mediums, presterilization manipulations and sterilization of plant material were conducted according to Rumynin & Slyusarenko (1989). The methodology of plant culture *in vitro* and preparation of samples for microscopic investigations follow Churikova et al. (1991) and Churikova & Barykina (1995, 2005). In all our experiments we observed direct morphogenesis, missing the callus stage (Fig. 1A, B, C). Anatomical sections of three leaf formations were analyzed using light microscope Micromed-3. Images of the sections were taken with light microscope Axioplan-2 Imaging equipped with digital camera AxioCam MRc and processed with Adobe Photoshop.

Histochemical analysis of essential cell substances as well as the secondary products of metabolism was carried out according to the recommendations of the 'Handbook of botanical microtechniques' (Barykina et al. 2004).

Results and discussion

Morphogenetic processes in cataphyll and medial leaf explants of lilies and hyacinths are generally similar. The first response on wounding is the formation of a protective film consisting of

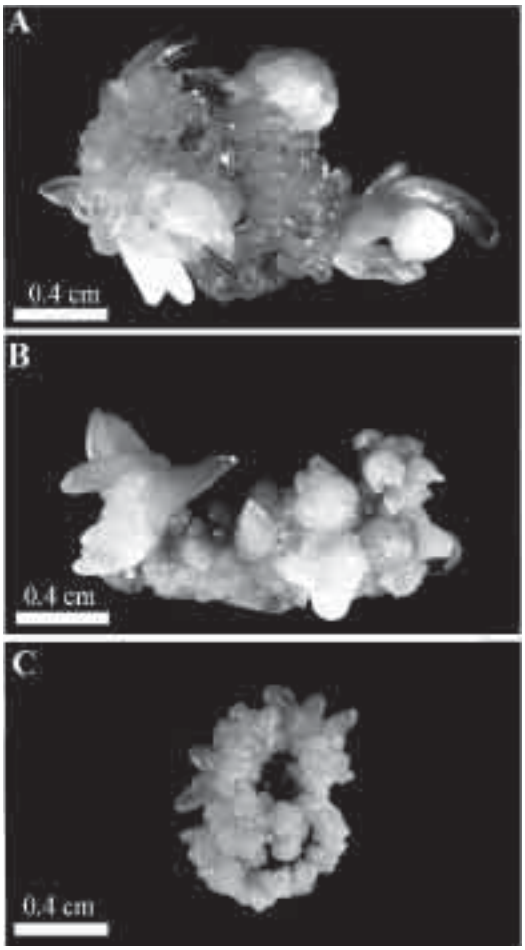

Figure 1. The appearance of primary explants of hyacinth with shoot rudiments developed *de novo*. A – bulb scale; B – leaf; C – peduncle.

destroyed cells and cell sap on the damaged surface. In explants of *L. regale* a wound plug consisting of several layers of periclinally divided cells is formed (Fig. 2A). Later the cell walls of their derivates suberize. Both protective film and wound plug protect living tissues of explants from outer infection and create favourable surroundings for displaying meristematic activity of cells as well.

At first meristematic activity in leaf explants of lilies and hyacinth is limited to 1–3 subepidermal cell layers of mesophyll which differ from other cells by their increased capacity to differentiation (Fig. 2B, C, E). However, the wave of cell divisions gradually spreads from the surface of explants to inwards and involves practically all living tissues, including parenchyma sheaths of vascular bundles (Fig. 2F) and cells of interbundle parenchyma (Fig. 2G). As a result, great meristematic areas are formed (Fig. 2D). Shoot rudiments and branched hydrocyte system (hydrocyte nodes and bundles) differentiate there. The latter one is a complex vascular system in structural and in functional respect (Churikova & Barykina 2005). Hydrocyte system functions for extraction of nutritive and biologically active substances from culture medium and for redistribution of inner resources within explants and for directing them to zones of growing processes. The formation of hydrocyte nodes (Fig. 4A, B, C) usually precedes the differentiation of shoot apexes. Rarely

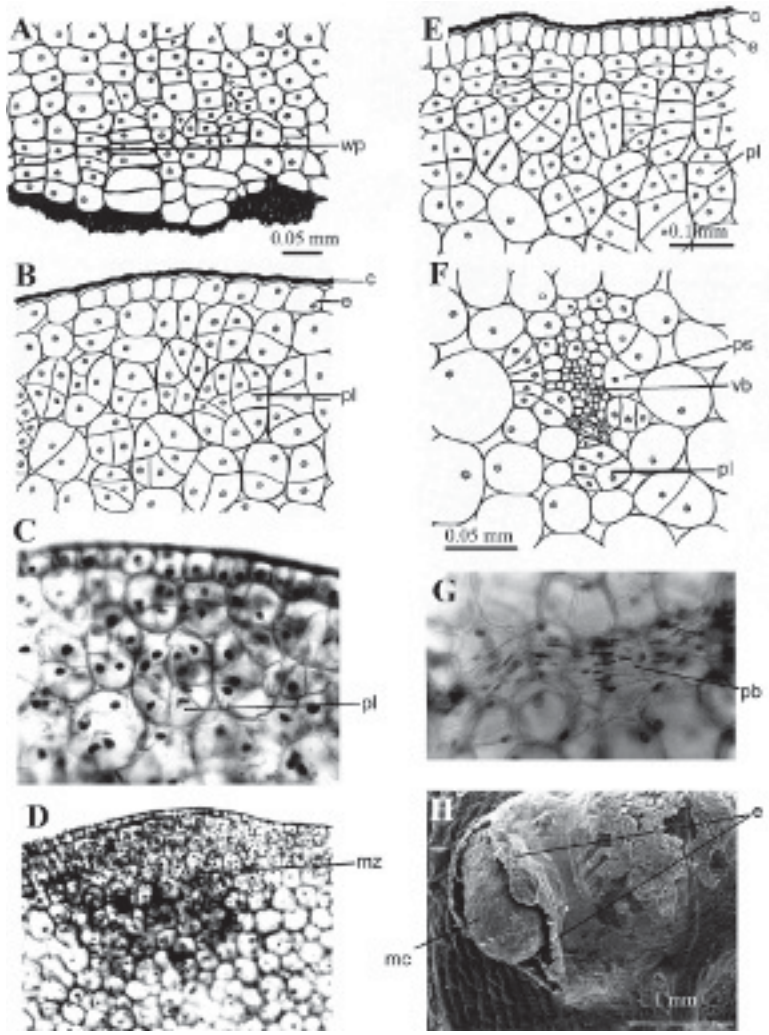

Figure 2. Morphological processes in explants of leaf nature in lilies (A, B, C, D, H) and hyacinth (E, F, G). A – wound plug formation; B, C, E – divisions of mesophyll cells and polyad formation; D – formation of meristematic zone; F – cell divisions of parenchyma sheath of vascular bundle; G – provascular bundle formation in interbundle zone; H – meristematic clump, which tears up the epidermis. e – epidermis; c – collenchyma; mc – meristematic clump; mz – meristematic zone; pb – provascular bundle; pl – polyad; ps – parenchyma sheath of vascular bundle; vb – vascular bundle; wp – wound plug.

they are formed in the bases of already developed shoot rudiments. The latter ones appear in meristematic zones usually endogenously as a result of several consecutive divisions and can be distinguished by their small cells. If adventive buds have 1–2 metamers, their own conductive system develops at their bases connecting with the conductive system of explants by hydrocyte nodes and branched bundles.

In all cases we have observed that epidermis, as a rule, doesn't take part in meristematic clump formation immediately. Its cells stretch out below the surface of subepidermal meristematic mounds and from time to time they undergo cell divisions exclusively by anticlinal cell walls. Under pressure of quickly grown endogenous clumps of meristems the epidermis tears up, its cells die and peel off (Fig. 2H). Relatively weak ability of the epidermis to differentiate is apparently

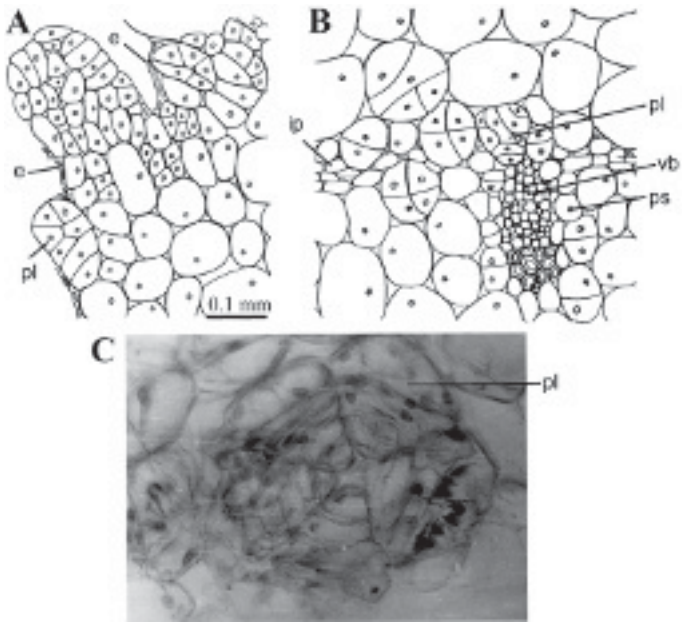

Figure 3. Morphological processes in explants of peduncle of hyacinth (A) and daffodil (B, C). A – intensive divisions of peripheric layer cells of primary cortex with the further formation of meristematic centers; B – cell divisions of parenchyma sheath of vascular bundle and C – in the pith. e – epidermis; ip – interbundle parenchyma; pl – polyad; ps – parenchyma sheath of vascular bundle; vb – vascular bundle.

due to its earlier and deeper specialization compared to the mesophyll and to the absence of sufficient nutritive substances as well.

Our analysis of morphogenesis in lilies confirmed the greater regeneration activity of inner bulb scales compared to outer bulb scales mentioned by Globa-Michailenko et al. (1986). This is evidently determined by a high level of soluble nitrogen and a low level of sugars in the former ones (Myodo & Kubo 1952). This is apparently true for hyacinth bulbs. Morphogenetic processes in the explants of cataphylls and medial leaves which differ in functions occur similar in lilies and hyacinths.

The ability to dedifferentiation and display of meristematic activity in explants of axillar nature (peduncle) of hyacinth and daffodil is demonstrated first of all by cells of non-damaged subepidermal layers and several outer layers of the primary cortex adjoined to them. After 7–10 days of cultivation they start to divide by periclinal cell walls. The polyads form relatively quick (Fig. 3A). During the further damage and lysis of their cell walls extensive clumps of meristem appear. Gradually cell divisions intrude into the explant. The inner layers of primary cortex, cells of vascular bundle sheaths (Fig. 3B) and even the cells of pith (daffodil) (Fig. 3C) turned out to be involved in this process. However, the meristematic potential of cells noticeably decreases from periphery to the center of peduncle. Within the peripheric meristematic zone some fine-celled groups are distinguished. They subsequently differentiate into shoot apexes. The cells of the primary cortex, out of which they spread, stretch in radial direction and bear rudiments of further buds. This process is especially vivid in hyacinth. Sometimes these spread cortex cells may undergo divisions and form a second, inner layer of meristematic centers situated below the former one. But, as a rule, their further development into rudiments of buds doesn't take place.

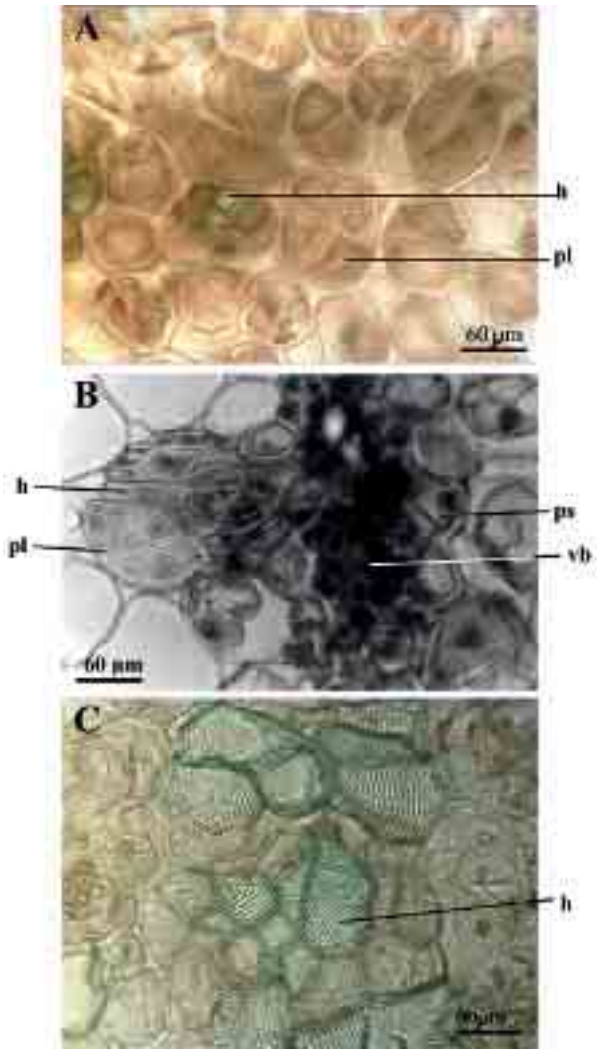

Figure 4. Initial stages of hydrocyte system differentiation in peduncle explants of daffodil (A), hyacinth (B) and lilies (C). h – hydrocyte; pl – polyad; ps – parenchyma sheath of vascular bundle; vb – vascular bundle.

At the end of the first month of cultivation the compound branched hydrocyte system differentiates in peduncle explants. Usually, formation of shoot apexes as mentioned above is preceded by the development of a hydrocyte system. Rarely the hydrocyte nodules appear in the bases of already formed bud rudiments. Probably this is due to the date of experiment (the end of April–May), when most of nutritious substances in peduncle have already been consumed for inflorescence formation. The absence of root development after 3.5 months of cultivation can be explained apparently by the small content of energy-rich substances in peduncle explants of hyacinth, a weakly developed hydrocyte system and a low level of endogenous auxin, too. The initiation of adventitious roots was observed only after transferring buds separated from explants to a special medium for rhizogenesis induction.

For microclonal propagation of plants not only leaf and axis, but also bud explants are widely used at the same time. The induction of axial bud formation is based on the raising of apical dominance. The use of bud with its compound system as initial explant leads to a different

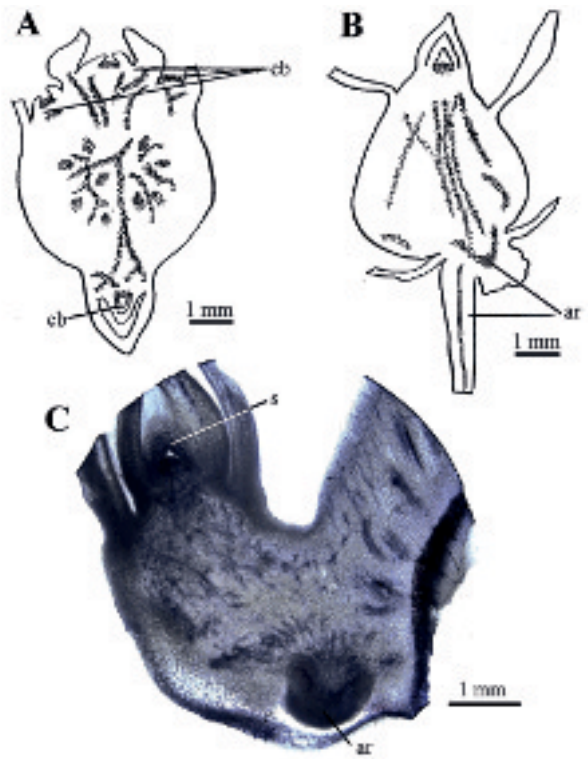

Figure 5. Intensive internal bud branching of primary (main) bud (A), *de novo* formed corms with rudiment of adventitious root in gladiolus (B), rooting shoot rudiment in bulb scale explant in hyacinth (C). ar – adventitious root; cb – collateral buds; s – shoot.

morphogenetic reaction *in vitro* compared to explants of leaf and axis. A week after placing gladiolus buds on the culture medium, axial middle (main) buds of two nearly opposite axillar complexes could be distinctly noticed. During further growth and bud development numerous initiations of additional buds on their right and left side takes place in the axial intercalary meristem zone. They undergo intensive internal branching leading to the formation of new collateral buds (Fig. 5A). As a result, complicated axillar complexes are formed. Their bud rudiments differ in size and degree of differentiation. Buds and corms, which form later, connect with each other by usual vascular bundles. The formation of a hydrocyte system doesn't take place there. The activation of axial meristem cells is the result of decapitation of initial maternal buds and the low content of cytokinins in the culture medium. In gladiolus adventitious roots initiate on the axis of corms (Fig. 5B), whereas in lilies, hyacinth and daffodil they initiate in explants of leaf and axis, too. Usually, this was noticed 14–27 days after the transfer of little corms on a special culture medium for the induction of rhizogenesis. The intensification of bud formation *in vitro* is accompanied by acceleration of shoot development. Regenerated plants planted out in spring started to blossom much earlier (after 6 months) than plants grown *in vitro*.

So, analysis of morphogenetic processes revealed that explants of certain parts of shoots (leaves, bulb scales (metamorphosed bases of cataphylls and medial leaves), leafless peduncles) from studied species of lilies, hyacinth, daffodil as well as explants of buds from gladiolus undergo, according to the terminology of Batygina (1990, 1991) gemmorhizogenesis (Fig. 5C). Practically all alive tissues of explants of leaf and axial nature can be involved into the formation of meristematic

centers. The greatest regeneration potential can be found in the first to third peripheral layers of mesophyll and primary cortex cells, which are photosynthetic active and rich in nutritious substances. Epidermis doesn't take part in the formation of meristematic centres because of its earlier specialization. Non-significant differences in morphogenetic reactions *in vitro* are due to a great extent to the morphological nature of the organ which is the source of explants and its function. To an even less degree it depends on taxonomical affiliation. The revealed similarity of leaf and axis explants in response to preparations, sectioning and growing *in vitro* may be an additional indication to regard the shoot as entire integrated plant organ. Tissues of its subordinated parts are of common origin.

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