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Regeneration of plants from leaves of *Chrysanthemum morifolium* Ram. cv. Bronze Bornholm in *in vitro* cultures

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Abstract

Plants were obtained from cultured *in vitro* leaves of *Chrysanthemum morifolium* Ram. cv. Bronze Bornholm. The leaves were inoculated on Murashige and Skoog medium (MS) supplemented with cytokinins (kinetin — KIN, zeatin — ZEA, 6-benzyloaminopurine — BAP) and auxins (2,4-dichlorophenoxyacetic acid — 2,4-D, α-naphtaleneacetic acid — NAA, 3-indolilacetic acid — IAA, p-fluorophenylalanine — PFA) in various combinations and concentrations. The most suitable medium was that one which contained 4 mg/l KIN, 2 mg/l NAA and 50 mg/l PFA.

INTRODUCTION

The method of *in vitro* culture is often used for vegetative propagation of ornamental plants. Under the influence of auxins and cytokinins present in the medium it is possible to regenerate various organs and tissues. Thanks to this phenomenon many plants can be obtained which are identical with a specified individual (Roest and Bokelmann 1973, Earle and Langhans 1974, Hauzińska 1975a, b).

Regeneration of leaves proceeds easily in some species belonging to the following families: *Solanaceae, Begoniaceae, Crassulaceae* and *Gesneriaceae*. The morphogenic potentials of leaves have been investigated in details in *Begonia*. The presence of cytokinin prooved to be indispensable

for bud initiation, whereas root formation was always related to the presence of auxin (Schraudolf and Reinert 1959, Writh 1959, Heide 1964, 1965, Bigot and Chlyah 1970, Bigot 1972, Bajaj 1972).

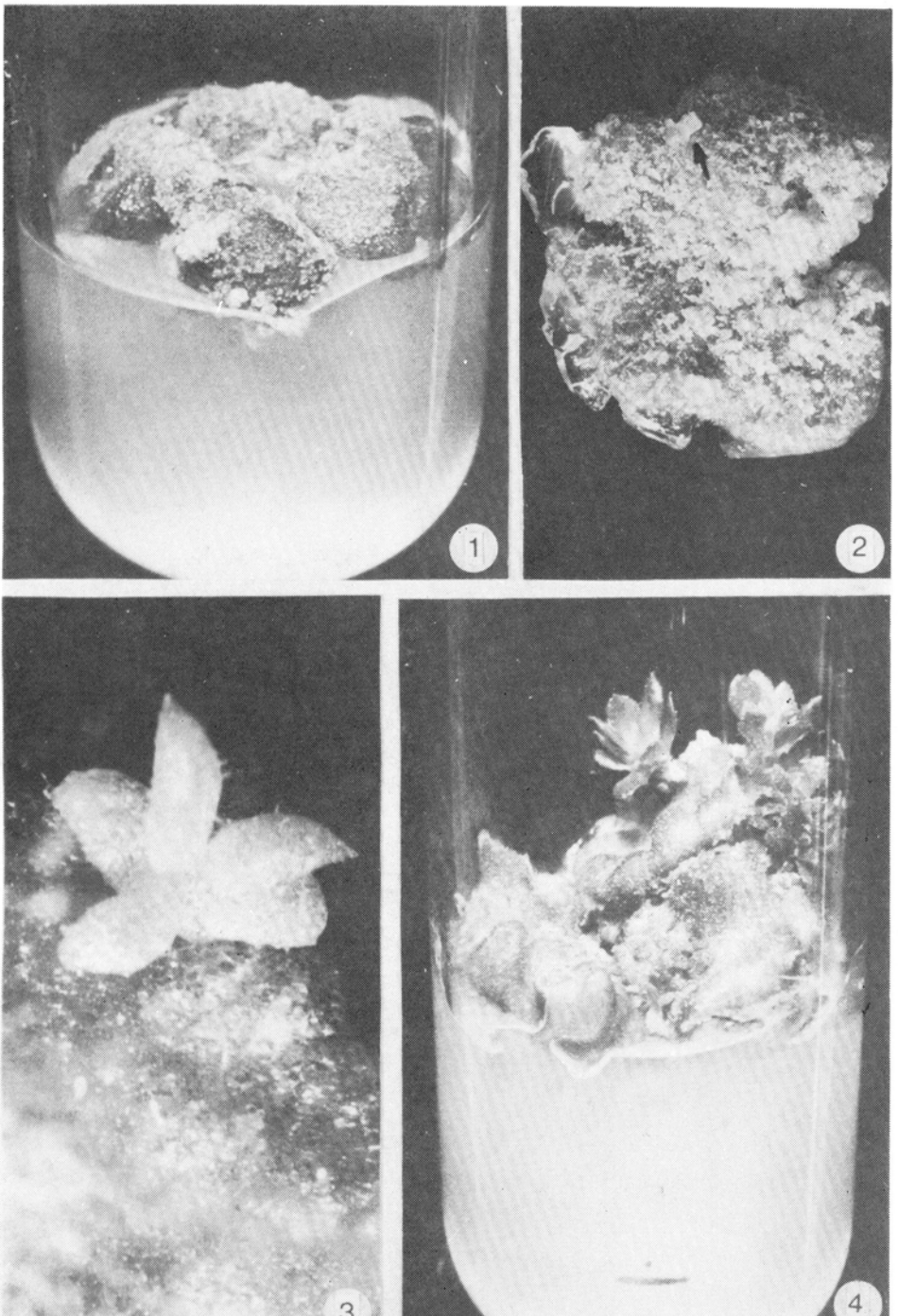
There are only few papers dealing with the regenerative potential of isolated petioles. Klimaszewska (1977) was able to regenerate petioles of 21 species representing 14 families but only petioles from two species regenerated roots and shoots, other formed callus and roots or only callus. Investigations have been already carried out on the *in vitro* cultured leaves of *Chrysanthemum*. Hill (1968) and Sangwan and Harada (1977) inoculated fragments of leaves of four varieties of *Chrysanthemum morifolium* Ram. but were not able to find a suitable medium for their regeneration. Bush et al. (1976) obtained plants from petals of the perianth, Broertjes et al. (1976) used X-rays to induce leaf organogenesis, and Roest and Bokelmann (1975) obtained buds from fragments of pedicles.

The aim of our investigations was to work out optimal conditions for the inducement of buds from cultured *in vitro* leaves of *Chrysanthemum morifolium* Ram. cv. Bronze Bornholm.

MATERIAL AND METHODS

Leaves of *Chrysanthemum morifolum* Ram. cv. Bronze Bornholm were isolated from plants which have developed from apical meristems cultured *in vitro*. Plants were propagated by dividing them into segments of which each one contained one node with one pair of leaves. Those segments were transferred to MS medium (Murashige and Skoog 1962) supplemented with 6% sucrose and 0.5 mg/l IAA. The explants were kept in the medium as long as plantlets reached size of about 10 cm. Later on leaves were cut off from three levals: a) the youngest leaves from the two upper nodes, b) slightly older ones from the two middle nodes and c) the oldest ones from the two lower nodes. Four leaves were transferred into one tube on different MS variants (Table 1). Alltogether 90 combinations of growth substances were used. On each variant of the medium 100 leaves were placed. A photoperiod (L:D = = 8:16) with the illumination of about 1500 lx and a temperature of $20 \pm 2^{\circ}C$ was used for all cultures.

Leaves at various stages of callus formation and differentiation of buds were fixed in FAA fixative and subsequently embedded in paraffin. Preparations 10-12 μm thick were stained with saffranin and light green.



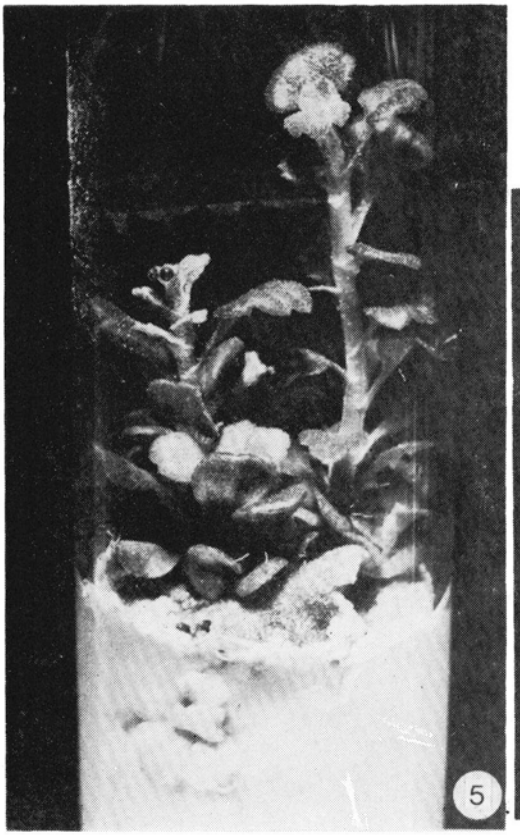
Chrysanthemum morifolium Ram. cv. Bronze Bornholm (MS medium + 4 mg/l KIN + 2 mg/l NAA + 50 mg/l PEA)

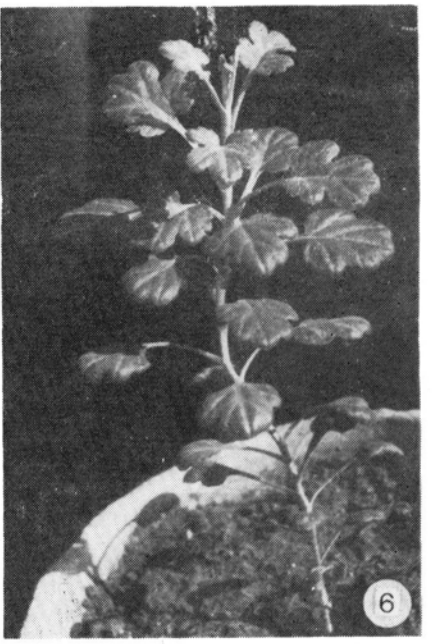
Fig. 1. Leaves after two weeks of culture covered with callus tissue. 2 ×

Fig. 2. Leaf after three weeks of culture with small buds (arrow). 6 ×

Fig. 3. Rosette-shaped plant on leaf surface after one month of culture. 10 ×

Fig. 4. Fully developed plantlets after six weeks of culture. 6 ×

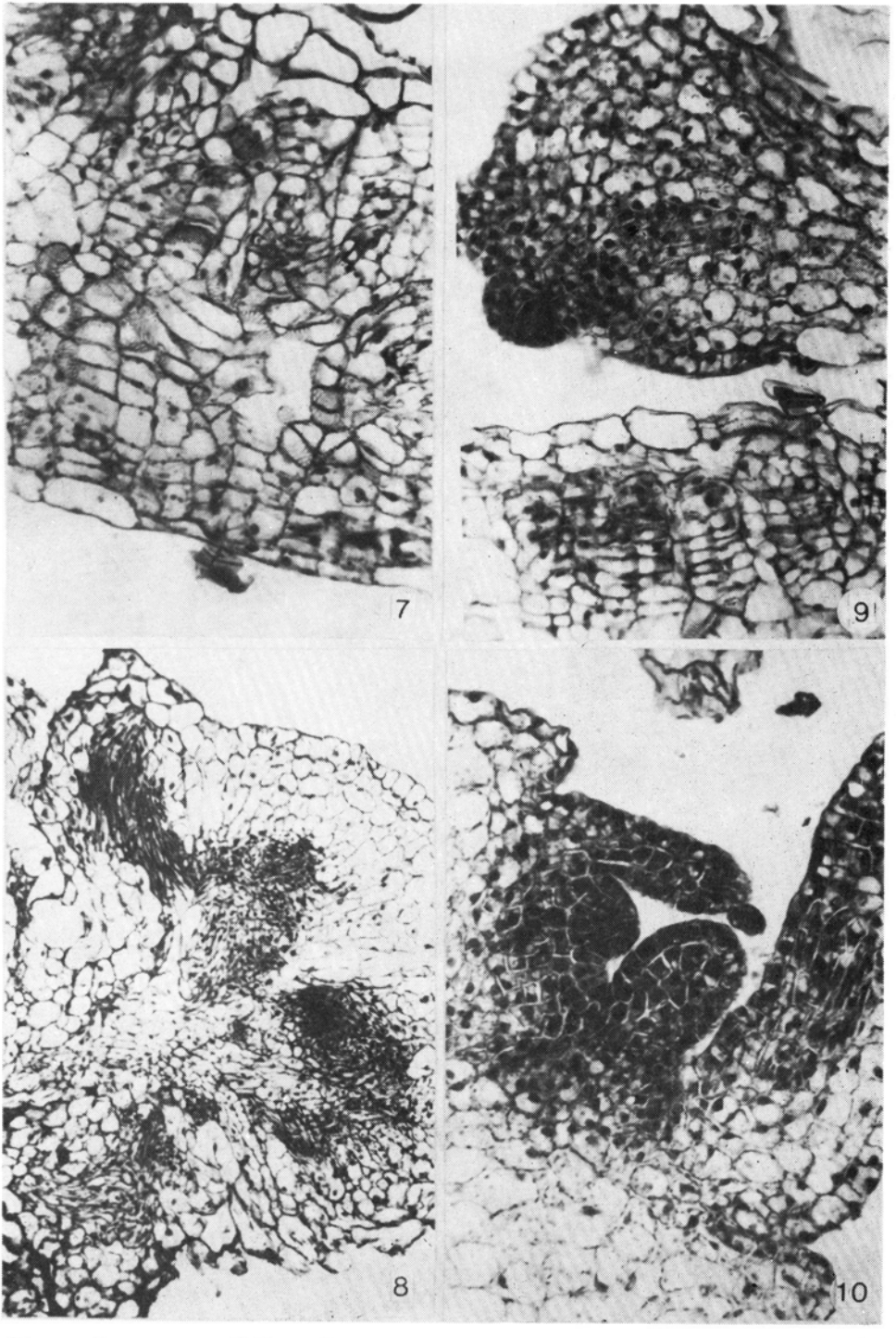




Chrysanthemum morifolium Ram. cv. Bronze Bornholm (MS medium + 4 mg/l KIN + 2 mg/l NAA + 50 mg/l PFA)

Fig. 5. Regenerated plants after two months of culture. 2 ×

Fig. 6. Plant which has developed on medium was transferred to soil. 1×



Chrysanthemum morifolium Ram. cv. Bronze Bornholm (MS medium + 4 mg/l KIN + 2 mg/l NAA + 50 mg/l PFA)

Fig. 7. Cross section through leaf after three weeks of culture; rows of cells formed after periclinal division of palisade cells and clusters of cells with reticulat and spiral thickening. 150 ×. Fig. 8. Callus tissue after three weeks of culture. In the centre clusters of meristematic cells. 150 ×. Fig. 9. Section through bud which developed on leaf surface after one month of culture. 450 ×. Fig. 10. Apex of shoot differentiating from the external cells of callus after one month of leaf

Table 1

MS medium supplemented with cytokinin and auxin used for *in vitro* culture of *Chrysanthemum* leaves

Cytokinin, mg/l		Auxin, mg/l		Other supplements, mg/l
KIN	1.0-5.0	2,4-D	0.25-1.0	c.h. — 200 ye.ex. — 500
KIN	1.0-10.0	NAA	1.0-5.0	PFA — 25.0-100.0
ZEA	0.5-2.0	NAA	0.5-1.0	—
BAP	0.5-2.0	2,4-D	0.25-1.0	—
BAP	1.0-5.0	IAA	0.25-1.0	—
BAP	1.0-5.0	NAA	0.5-2.0	—

KIN — kinetin, ZEA — zeatin, BAP — 6-benzylaminopurine, 2,4-D — 2,4-dichlorophenoxyacetic acid, NAA — α-naphtaleneacetic acid, IAA — 3-indolilacetic acid, PFA — para-fluorophenylalanine, c.h. — casein hydrolysate (acid), ye.ex. — yeast extract

RESULTS

The high rate of regeneration of buds and roots from *Chrysanthemum* leaves was achieved with the medium supplemented with 4 mg/l KIN + + 2 mg/l NAA + 50 mg/l PFA. In the first week of culture leaves started to produce callus. At first callus appeared at the base of leaf and during the next few days it covered the whole leaf surface (Fig. 1). After two weeks of culture buds started to emerge from the callus tissue (Fig. 2). Concomitantly with bud formation also very short roots appeared. Large number of roots were produced along the main vascular bundle of the leaves. After one month of culturing leaves small plantlets developed from calluses (Fig. 3). During the next twoo weeks those plantlets attained a height of 3 cm. On average 40% of the inoculated leaves produced plantlets and usually from one leaf grew 10-15 small plantlets (Fig. 4). It has to be stressed that on the above mentioned medium all leaves formed calluses but buds differentiated only in 40% of the explants. However, roots were produced in much higher rate, usually in 70% of the inoculated material.

Small plantlets after two months of culture (Fig. 5) were transferred to vermiculat and subsequently to pots containing soil (Fig. 6). On other media leaves also produced calluses but shortly they became brown and died. No buds or plantlets developed from those calluses. However, buds and plantlets developed from the base of some leaves after 3 weeks of culture on the following media: MS + KIN (1-2 mg/l) + 2,4-D (0.25--0.5 mg/l) and on MS + BAP (1 mg/l) + 2,4-D (0.25-0.5 mg/l). When MS medium was supplemented with ZEA (0.25-2.0 mg/l) + NAA (0.5 mg/l) or with ZEA (2 mg/l) + 2,4-D (0.25 mg/l) then at the base of the leaves

small rosette-shaped plantlets appeared after three weeks. In order to induce the development of roots plantlets were transferred to medium MS supplemented with KIN (0.5 mg/l) and NAA (1 mg/l). Irrespectively of the age of inoculated leaves their regenerative potencies were alike.

Callus developed from parenchymatic cells, both from spongy and pallisade parenchyma. This tissue was composed of parenchymatic isodiametric cells as well as of cells of meristematic character (Fig. 8). Within the callus large clusters of cells with reticulate and spiral thickenings were observed (Fig. 7). Calluse grew actively and most of the meristematic regions differentiated into buds (Fig. 9, 10).

DISCUSSION

We found a suitable medium for the inducement of buds from leaves of *Chrysanthemum morifolium*. High percentage of explants had developed calluses and multiple shoots. The addition of KIN and NAA in a ratio of 2:1 played very important role in the induction of organogenesis. The application of KIN and NAA in such a ratio enabled the simultaneous formation of shoots and roots. It is worth to note that when the basic MS medium was supplemented with KIN and NAA in a ratio of 2:1 and with 25-50 mg/l PFA organogenesis took place on the whole leaf surface, whereas without PFA only single plantlets at the base of leaf regenerated. Thus PFA seemed not only to induce the development of callus but, what is important, favoured the formation of buds.

It has been known for a long time that for the regeneration of various organs in the *in vitro* culture it is necessary to supplement the medium with cytokinin which induces cell division and at the same time stimulates the synthesis of ribonucleic acids. Schraudolf and Reinert (1959), Writh (1959), Heide (1964, 1965), Bigot and Chlyah (1970), Bigot (1972) had in details analysed the process of regeneration of leaves of *Begonia*. Those authors found that the cytokinins strongly stimulated bud formation, while auxins only stimulated the rhizogenesis. From the investigations carried out by Bajaj (1972) on the regeneration of *Torenia fournieri* leaves, as well as from the earlier informations published by Skoog and Miller (1957) on callus obtained from tobacco pith it appears that the growth of tissue and differentiation of organs depend on a subtle balance between auxins and cytokinins.

The results of our experiments on the regeneration of leaves of *Chrysanthemum* also indicate that the addition of KIN and NAA at a strictly determined ratio of 2:1 is very important for the induction of organogenesis. Similariy as Bush et al. (1976) we have found that regeneration of plants from *Chrysanthemum* leaves always took place through

the formation of callus. Direct development of buds from leaf was never noticed. In contrast, Broertjes et al. (1976) have shown that epidermis of petioles was able to produce buds. In general, in order to regenerate plants from leaves it is necessary to supplement the basic medium with cytokinin and auxin. However, as it was shown earlier by Wareing and Philips (1978), the reaction of explants to such medium mainly depends on the species, even on the variety, what indicates that the regenerative potential is genetically determined.

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Regeneracja roślin z liści Chrysanthemum morifolium Ram. cv. Bronze Bornholm w warunkach in vitro

Streszczenie

W kulturach *in vitro* otrzymano rośliny na drodze regeneracji liści *Chrysanthemum morifolium* Ram. cv. Bronze Bornholm. Liście wykładano na pożywkę agarową wg Murashige i Skooga (MS) wzbogaconą w cytokininy (kinetynę — KIN, zeatynę — ZEA, 6-benzyloaminopurynę — BAP) i auksyny (kwas 2,4-dwuchlorofenoksyoctowy — 2,4-D, kwas α-naftalenooctowy — NAA, kwas 3-indolilooctowy — IAA) w różnych kombinacjach i stężeniach. Optymalnym podłożem dla regeneracji liści była pożywka zawierająca 4 mg/l KIN, 2 mg/l NAA i 50 mg/l para-fluorofenyloalaniny (PFA).