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Morphogenic effect of colchicine in *Cichorium intybus* L. root explants cultured *in vitro*

Bennici* Andrea, Silvia Schiff and Bruno Mori

Department of Plant Biology, University of Florence, P.le delle Cascine 28, 50144 Florence, Italy.

Abstract — A simple and efficient protocol for *in vitro* shoot regeneration from storage root explants of witloof chicory (*Cichorium intybus* L.) has been used to study the effect of colchicine at different concentrations, and colchicine in combination with kinetin or 2,4-D or with 2,4-D and kinetin, in comparison with kinetin and control (no treatment), upon this type of morphogenic event. The results demonstrated a strong stimulatory effect of colchicine at low concentration on shoot regeneration frequency when compared to the number of shoots formed in the control. This effect was comparable to that of kinetin, a growth regulator that induces shoot differentiation. By contrast, the presence of 2,4-D completely inhibited shoot regeneration. Colchicine at high concentration did not induce shoots, and caused the complete death of the explants.
A carefully histological analysis of the explants during the *in vitro* culture period showed the formation of numerous meristematic zones (precursors of adventitious bud and shoot development) in the parenchyma mass; these meristems were present only in the explants subjected to the colchicine and to the other treatments able to stimulate shoot differentiation.
Nuclei of different sizes were observed in the explants treated with colchicine at low dose, the largest, probably, polyploids. Polyploid nuclei, in fact, were found in the regenerated plants.

Key words: *Cichorium intybus,* colchicine, *in vitro* culture, morphogenic stimulation

INTRODUCTION

The importance of polyploid plants in agriculture is well known (Lewis 1980; Gao *et al*. 1996; Koutoulis *et al*. 2005). Colchicine has been largely used to alter ploidy level frequencies in cell populations, either *in vivo* (Levan 1939; 1942; Eigsti and Dustin 1955; Drobets and Pestova 1980; Roy *et al.* 2001; Koutoulis *et al*. 2005) or *in vitro* (Gmitter *et al*. 1991; Gao *et al*. 1996; Pinheiro *et al*. 2000; Rose *et al.* 2000; Kadota and Niimi 2002), to obtain tetraploid plants, starting with diploid material. In fact, polyploid plants usually present larger and thicker leaves, stems or roots, and also flowers, seeds and fruits, and, then, a greater yield. However, this technique can also generate mixoploid cells; therefore, chimera plants consisting of diploid and tetraploid cells/tissues may develop (Barnabas *et al*. 1991; Koutoulis *et al*. 2005; Roy *et al.* 2001). Another important use of colchicine concerns its application for chromosome doubling in microspore derived haploid embryos to produce fertile homozygous diploid plant lines (Barnabas and Kovacs 1990; Barnabas *et al*. 1999; Zaki and Dickinson 1995).

These colchicine effects are based on the inhibition of the mitosis. This is due to the binding of colchicine to tubulin, one of the main constituents of microtubules and, therefore, of the nuclear spindle, the development of which is hampered (Eigsti and Dustin 1955; Olmsted and Borisy 1973).

In this context, another possible and interesting effect of this drug seem to be a promotional influence on cell division, although, contrasting results have been reported on this subject (Dustin and Chodkowski 1938; Miszurski and Doljanski 1949; Delcourt 1938; 1940; Eigsti and Dustin 1955).

However, more recently, Barnabas and Kovacs (1990) and Barnabas *et al.* (1999) on *in vitro* anther culture of *Triticum aestivum* and *Zea mays*, respectively, have found a positive effect of colchicine on pollen callus induction and plant regeneration. A similar effect has also been shown on *in vitro* anther and isolated microspore cultures of *Brassica napus* where this compound stimulated

* Corresponding author: phone: ++39 055 3288270; fax: ++39 055 360137; e-mail: andrea.bennici@unifi.it

cell division and embryogenesis (ZAKI and DICKINSON 1995).

In the previous years protocols have been developed in our laboratory for the regeneration of plantlets from storage root explants of witloof chicory (*Cichorium intybus* L.). In this material shoot regeneration at elevated levels occurred not only on a medium lacking auxins and cytokinins (CAFFARO *et al*. 1982; PROFUMO *et al.* 1985), but also in distilled water solidified with agar (BENNICI 1985).

Therefore, we have used this last *in vitro* very simple but efficient morphogenic system for a research with the aim to ascertain the effect of colchicine on shoot regeneration, taking into account also its action on ploidy level modification.

MATERIALS AND METHODS

Cichorium intybus L. (cv "Sweet chicory of Soncino") storage roots were used as experimental material. The roots, previously washed under tap water, were surface sterilized by immersion in 30% commercial hypochlorite (7% active Cl^-) for 10 minutes. After four washes in sterile distilled water, the roots were cut into slices from which disk explants (10 mm diameter and 5 mm thickness) were excised along the cambial ring. This meristem remained localized at the middle zone of each explant. All the explants, collected at random from the part of the roots comprised between the base and the middle zone of these, were placed on the surface of 30 ml distilled water solidified with 0.8% (w/v) Difco Bacto agar (in 100 ml Erlenmeyer flasks, two explants per flask), supplemented with colchicine (SIGMA), or colchicine and growth regulator(s), or a growth regulator only. Two distinct experiments were carried out. As far as the first experiment, the explants were subdivided into 4 groups and subjected to the following treatments: control (only water), 0.01, 0.005 and 0.001% (w/v) colchicine. For the second experiment, 6 groups of explants were used according to these treatments: control, only 0.001% colchicine, 0.001% colchicine plus 0.5 μM kinetin, only 0.5 μM kinetin, 0.001% colchicine plus 5 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 0.001% colchicine plus 5 μM 2,4-D and 0.5 μM kinetin. Every group consisted of 40 explants; they were maintained in 100 ml Erlenmeyer flasks for 40 days. Scoring for explant behaviour and shoot regeneration was made 25 days after sowing. The frequency of regeneration for each treated group was calculated in terms of increase or decrease of the respective final total number of shoots in comparison with that of the control. This difference was expressed as percentage making the respective control shoot number equal to 100. In these experiments the flasks were completely randomized, then obviating any bias during the scoring.

Part of the resulting regenerated shoots, excised at the base (5-8 mm long), were rooted in 30 ml MURASHIGE and SKOOG (1962) half-strength basal solid medium containing 5 μM indole-butyric acid (IBA) in Magenta (SIGMA) vessels.

All the cultures were incubated in a growth room at 25±1°C under 16 hours illumination (35 μmol m^{-2} s^{-1} light intensity) provided by Sylvania Daylight (F36W/154-ST) cool fluorescent tubes.

The differen gelled solutions (and controls) were sterilized by autoclaving at 120 °C for 20 minutes and 0.1 MPa.

Samples of plantlets with well developed roots, after acclimation into pots containing sterile peat under transparent plastic at ambient temperature (about 25 °C), 80% relative humidity to prevent desiccation, and in the same light condition above reported, were transferred to soil and maintained in a glasshouse for 2-3 months.

For histological observations of the explants treated according to the protocols mentioned, pieces of these, chosen at random at 2-3 day intervals during the culture period, were processed as follow. Fresh root slices (60-80 μm thickness), pretreated with 50% ethanol and rehydrated, were stained with 0.1 μg/ml DAPI (CLARK 1981).

For karyological analysis of regenerated plantlets, root apices, pretreated with 0.2% colchicine at 25 °C for 4 hours, were fixed in ethanol-acetic acid 3:1 (v/v) and prepared as squashes stained by Feulgen or haematoxylin technique. The slides, after dehydrated in an alcohol series, were made permanent with DPX mountant (BDH).

RESULTS

First experiment. - All control root explants, after 6-10 days of culture, responded by producing a well visible whitish or light green wound callus on their air exposed surface. Contrarily, the explants treated with colchicine at 0.001 and 0.005% formed only a light layer of dedifferentiated tissue. Either the control or the explants grown in presence of colchicine at the lowest doses produced adventitious buds on their upper part. Bud initiation was observed after 10-13 days of culture. These buds developed into shoots (without roots) during the next one-two weeks. Each explant formed two-five plantlets. The

shoots in the same explant exhibited different growth capacity. In general, one or two plantlets overgrew the others. However, when the final number of regenerated shoots was evaluated, strong differences between control and treated explants, as well as among the different colchicine treatments, were found. Considering the total number of shoots regenerated in presence of 0.001% colchicine in comparison with the control, an increase of 95% resulted. By contrast, the colchicine at 0.005 and 0.01% strongly reduced the formation of the shoots causing a decrease of 93% and a complete absence of regeneration, respectively (Table 1).

Table 1 — Total number of regenerated shoots in *Cichorium intybus* explants after 25 days of culture in presence of colchicine

Colchicine concentration (%)	No. of regenerated shoots
No colchicine	143
0.01	0
0.005	10
0.001	279

Second experiment. - Also in this case the stimulatory effect of colchicine at the lowest dose upon shoot regeneration was confirmed; in fact, the frequency of regenerated shoots, when compared to the control, showed an increase of 60%. However, this increase, in comparison with the same treatment type of the previous experiment, was lower. The combination of kinetin with colchicine was also very effective in causing shoot regeneration (93% regeneration increase), likely the effect of colchicine observed in the first experiment. The positive role of kinetin was observed also when this compound was used without colchicine, inducing shoot differentiation with the same increase induced by colchicine alone, above reported (60%) (Table 2). Also in these regenerating explants the shoots for each explant varied from 2-3 to 5-6. The same callusing effect of colchicine, before reported, was observed.

Table 2 — Total number of regenerated shoots in *Cichorium intybus* explants after 25 days of culture in presence of only colchicine or colchicine and growth regulator(s) or growth regulator(s) alone.

Treatment	No. of regenerated shoots
No treatment	151
0.001 (%) C	243
0.001 (%) C + K	292
0.001 (%) C + 2,4-D + K	0
K	241
2,4-D + K	0

C: colchicine; K: kinetin (0.5 μM); 2,4-D: 5 μM.

On the contrary, shoot regeneration was completely inhibited by 2,4-D, either when used in combination with colchicine or with colchicine and kinetin (no regeneration, respectively). This substance induced only an abundant surface callus in the explants, except in presence of colchicine.

The histological analyses clearly supported the presence or the absence of the organogenic response in the explants. Considering that every regeneration event in a primary explant or in a callus is preceded by the formation within the tissue mass of meristematic centers or nodules which can develop, successively, into buds or root apices, the occurrence of these meristems was effectively found in the explants, according to the organogenic treatments above reported.

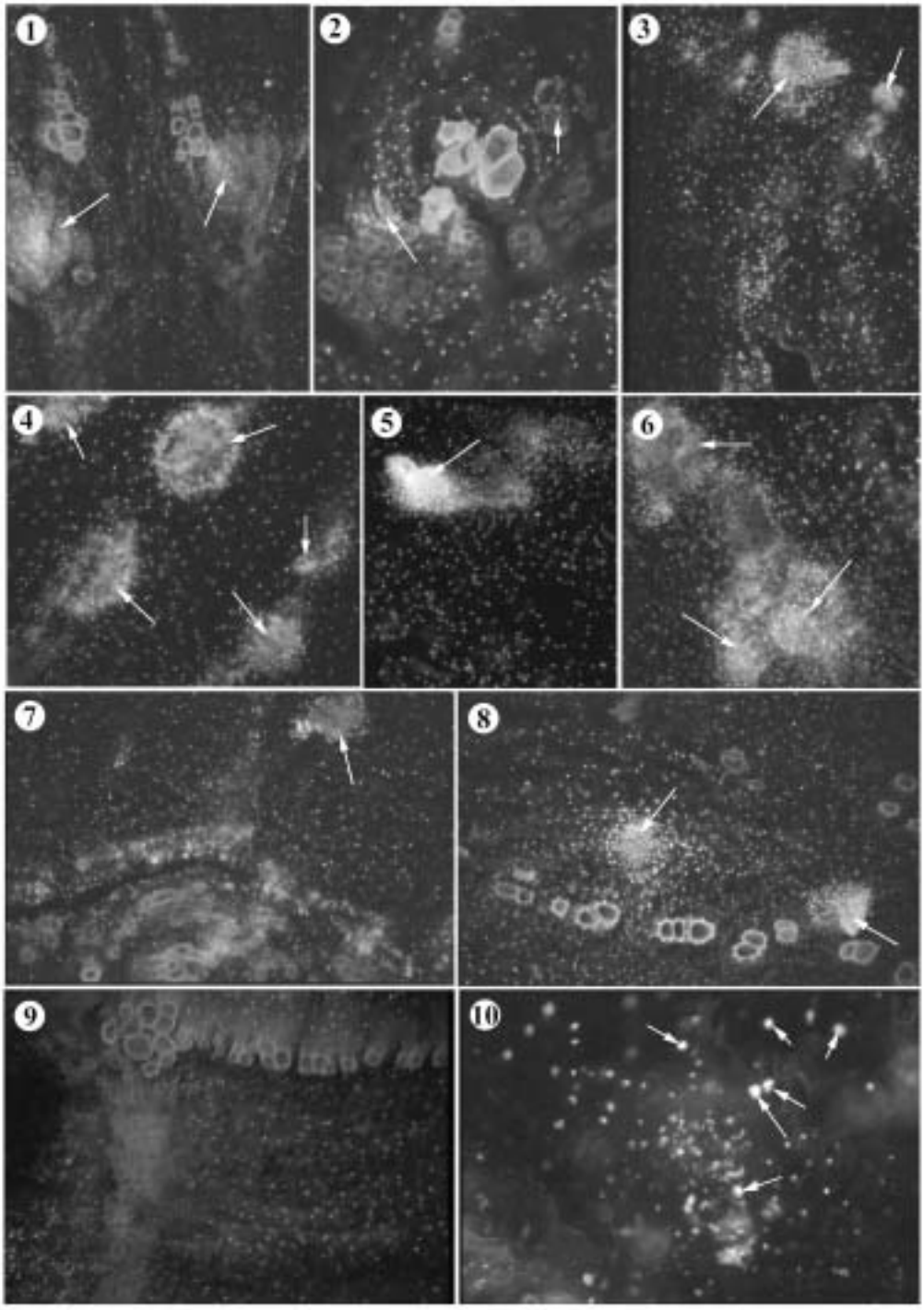
In fact, all control explants exhibited numerous meristematic nodules, especially near the outer upper callusing part (Figs 1, 2), as well as the explants treated with 0.001% colchicine (Figs. 3, 4). These nodules, scattered among the parenchyma cells, were easily visible because formed by very small cells of meristematic type. Similar pictures were found also in the explants grown in presence of colchicine and kinetin (Figs. 5, 6), or kinetin (Figs. 7, 8). Instead, all the explants treated with 2,4-D did not show any meristematic center formation (Fig. 9).

In regard to the role of colchicine in the polyploidy induction, nuclei of different size, some of which very large, were observed in the explants grown in presence of 0.001% colchicine (Fig. 10).

The sample of regenerated plants when transferred to soil were able to grow exhibiting normal morphological characteristics and a survival rate of 100%.

The cytological analysis of root apices collected from samples of plantlets grown in presence of 0.001% colchicine or colchicine with kinetin revealed diploid (2n=18) and tetraploid chromosome numbers in their meristematic cells, in different ratios. On the contrary, polyploid nuclei were not found in control plants.

After 25-30 days of culture, the explants grown in presence of colchicine at the lowest dose exhibited initial necrosis symptoms which, successively, increased causing the arrest of callus growth and shoot regeneration. The colchicine at the concentrations higher than 0.001% induced the complete death of the explants.



Figs. 1, 2 — Control explants showing meristematic zones (arrows); xylem elements are present (10×).
Figs. 3, 4 — Explants grown in presence of 0.001% colchicine (first and second experiment, respectively): note the large nodular meristems (arrows) (10×).
Figs. 5, 6 — Colchicine plus kinetin treated explants: well formed meristematic centers (arrows) are present (10×).
Figs. 7, 8 — Explants treated with kinetin: meristematic zone are clearly visible (arrows); xylem elements are present (10×).
Fig. 9 — Presence of 2,4-D in the cultured explants: note the complete absence of meristems in the parencyma tissue, where xylem cells are present (20×).
Fig. 10 — Explants grown in presence of 0.001% colchicine: large, probably polyploid nuclei (arrows), other smaller nuclei, are present in the parenchyma mass (20×).

DISCUSSION

Colchicine, an highly poisonous alkaloid, originally extracted from *Colchicum autumnale*, is used in medicine, especially for the treatment of gout. However, the greatest importance of this drug is due to its large botanical and agricultural use for inducing polyploid plants because these show more desiderable characters than normally diploid parents.

An important question concerning colchicine still remains its possible stimulation of cell division/growth. This fact is, clearly, in contrast with its well known inhibitory effect on mitosis (see Introduction).

The results of our investigation show that the continuous administration of this compound to the storage root explants of *Cichorium intybus* promotes: i) the dedifferentiation of parenchyma cells which "differentiate" into meristematic cells which form many meristematic nodules or "meristemoids" (Torrey 1966); ii) the further progression of these meristems towards the development of adventitious buds and shoots. It is evident that during these processes the pattern and frequency of cell division must be involved.

In this context, numerous studies reported positive effects of colchicine on growth and cell division. A stimulatory effect of mitoses has been described in human liver cells (Miszurski and Doljanski 1949) and in the regenerating tail of tadpoles of *Xenopus laevis* (Luscher 1946a; b). Other evidences of a mitotic exicitation following treatments with colchine were supported by several works of Dustin Sr. (1936; 1939; 1943). In tissue cultures of *Helianthus tuberosus* small doses of colchicine enhanced the action of auxin (indole-acetic acid) because the cells divided more actively; instead, at higher doses colchicine leaded to C-mitoses and inhibited cell multiplication (Martin 1945). This report is a clear example, among other similar cases, of a synergism between colchicine and another substance (Lettrè 1950; 1951; Deysson 1945; 1949). This phenomenon of synergism may be involved also in the response of *Cichorium intybus*. In fact, the great capacity of *Cichorium* to regenerate shoots without growth regulators suggests an endogenous capacity or potentiality already existing in the explants toward this type of morphogenic event. This caulogenic capacity or competence may be due to the presence of a favourable hormonal condition, i.e., a low auxin/kinetin ratio (Profumo *et al.* 1985)

However, in regard to this particular positive action of colchicine upon morphogenic processes in plant materials, only few reports exist (Barnabas and Kovacs 1990; Barnabas *et al.* 1999; Zaki and Dickinson 1995). Although in these works the morphogenic systems used are different from our, because they consisted in an embryogenic stimulation starting from anther or microspore cultures, they show many analogies with our investigation, such as the experimental procedure consisting in the administration of colchicine for a long period through the culture medium; similarly the drug seems to be effective only if below a determined threshold affecting spindle function. Moreover, a close correlation between drug concentration and embryogenic (or in our case organogenic) induction has been observed. Surely, a relationship between dose/effect must be very important. In fact, although this relationship cannot be the same if the plant systems are different, it is interesting to note that high concentrations of colchicine in all these systems resulted in a dramatic decline in morphogenesis.

The result obtained with kinetin treatment if compared with those produced by only colchicine could be in agreement with the idea of an hormonal or hormone-like action of this alkaloid, which was put forward by some botanical works (Havas 1938; 1939; 1940). The kinetin, as other cytokinins, is a causative agent in bud differentiation *in vitro* (Profumo *et al.* 1985; Anzidei *et al.* 2000). By contrast, 2,4-D, in accordance also with our present observations, induces growth in terms of callus, but strongly inhibits shoot formation (Lupi *et al.* 1985; Profumo *et al.* 1985). The effect of this auxin seems to prevail over those of colchicine and /or kinetin.

Moreover, it is possible to hypothesize a relationship between these morphogenic effects of colchicine and a stress condition resulting by the poisonous action of the drug itself, as suggested by Gmitter *et al.* (1991) in a work on sweet orange cultures, and Zaki and Dickinson (1995) that mention the induction of stress proteins. A relationship between the stressing action of colchicine and alterations of cell behaviour has been reported also by Ferguson (1952; 1953), Lits (1934), Cavallero (1939a; 1939b; 1947) in different animal or human systems.

In conclusion, besides the genome doubling effect of colchicine, a possible stimulatory influence of this compound on plant cell-tissue growth and developmental processes must not be undervalued. Undoubtedly, further investigations are necessary to resolve the conflicting stories of "mitotic arrest" or "arrest after mitotic stimulation" (Eigsti and Dustin 1955), and discover the bio-

logical basis of the morphogenic action of this alkaloid so far observed.

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