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MORPHOLOGICAL AND HISTOLOGICAL ASPECTS OF 2,4-D EFFECTS ON RAPE EXPLANTS (*BRASSICA NAPUS* L. CV. KANA) CULTURED IN VITRO

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The effect of different durations of exposure to 2,4-D on hypocotyls and cotyledons cultured in vitro was studied in *Brassica napus* L. cv. Kana. Organogenesis or callogenesis depended on the duration of explant exposure to MS medium supplemented with 2 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid). Short treatment (1 and 3 days) with 2,4-D resulted in rhizogenesis on hypocotyls (100% and 98% of explants, respectively) and cotyledons (80% and 54% of explants, respectively). Adventitious shoots formed sporadically, with the highest frequency (14% of explants) on hypocotyls cultured 3 days on MS supplemented with 2,4-D and then transferred to hormone-free medium. Histological analysis clearly indicated that the basal part of hypocotyls is involved in root formation and callus production, and the apical part for shoots. Meristematic sites originated from groups of cells in the cortex layer (including cells of the endoderm), but the procambium, phloem and pericycle also showed meristematic activity. The present study indicated that the response of explants cultured on media containing 2,4-D at constant concentration depends on the duration of explant exposure to growth regulator.

Key words: *Brassica napus* L., 2,4-D, regeneration, callus, histology.

INTRODUCTION

Auxin is a phytohormone involved in the control of various aspects of growth and development in higher plants (Rück et al., 1993). The regulated, differential distribution of auxin influences many processes including organogenesis and meristem patterning. Because of its multiple roles, auxin displays some characteristics of both a hormone and a morphogen (Friml, 2003).

The auxin signal is received by plant cells and rapidly transduced to a wide variety of responses in the growth and development of plant organs. These include changes in the direction of growth, shoot and root branching, and vascular differentiation (Leyser, 2001).

According to Petrášek et al. (2002), the division and growth of most types of plant cells cultured in vitro require an external source of auxin. In cultures, the ratio of external to internal auxin concentrations is essential for regulation of the phases of the standard growth cycle. The type of auxin used in the medium influences culture morphology (Hofmann et al., 2004). Synthetic auxins such as 2,4-dichlorophenoxyacetic acid (2,4-D) are essential constituents of culture media. They are used in cereals, for example, for callus induction and maintenance (Pellegrineschi et al., 2004) or for induction of somatic embryogenesis if applied at a higher concentration (Vikrant and Raskid, 2003).

Brassica napus (oilseed rape) has been the object of extensive tissue culture studies. Economically, *B. napus* is among the most important oil crops both for food and for industrial purposes. Many of the *Brassica napus* varieties, especially those with superior properties such as high yield, disease resistance and high quality, are fundamental material in breeding programs (Hu et al., 1999). Some authors have described the effect of plant growth regulators on culture of *Brassica* species (Rogozińska and Drozdowska, 1980; Klimaszewska and Keller, 1985; Bagniewska-Zadworna, 2001), but precise data on the effect of the duration of exposure to the phytohormone 2,4-D are scarce. It is known that plant regeneration capacity may vary significantly between species, as well as between genotypes within species (Jourdan and Earle, 1989).

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The aim of the current study was to examine the effect of different durations of exposure to a constant 2,4-D concentration (2 mg/l) on hypocotyl and cotyledon explants of *Brassica napus* cv. Kana. This Polish cultivar has important properties such as frost resistance and high yield, but no data about its growth characteristics in culture in vitro are broadly available. The concentration of 2,4-D was chosen on the basis of previous reports indicating that 2 mg/l 2,4-D is optimal for callus induction in *Brassica* (Khan et al., 2002) and in *Zea* (Bronsema et al., 1997; Emons et al., 1993).

MATERIALS AND METHODS

PLANT MATERIAL

Seeds of *Brassica napus* L. cv. Kana (Hodowla Roślin Strzelce, Inc., Poland) were sterilized by soaking in 70% ethanol for 60 sec and in Ace commercial bleach (diluted 1:1 with distilled water) for 12 min, followed by three rinses with sterile distilled water. The seeds were then placed in 10 cm Petri dishes on moistened sterile filter paper and incubated at 25 ± 3°C in the light. Fragments of hypocotyls (~5 mm in length) and cotyledons isolated from 5-day-old seedlings were used as explants.

CULTURE CONDITIONS

MS medium (Murashige and Skoog, 1962) was the basal medium. The media were solidified with 0.7% Difco BactoAgar and adjusted to pH 5.7–5.8 with 0.1 N HCl or NaOH prior to autoclaving at 121°C and 1.05 bar for 21 min. Explants were cultured on induction medium (IM) supplemented with 2 mg/l 2,4-D for 1, 3, 5, 7, 14, 21 or 28 days, and then transferred to hormone-free medium (GM) for the remaining days left, if any, in a total 28 days of culture (Tab. 1). All cultures were grown under a 16 h photoperiod (cool-white fluorescent tubes 60–90 µmol photons $m^{-2}s^{-1}$) at 25 ± 3°C.

Five explants (hypocotyls or cotyledons) per Petri dish were inoculated, with at least three replicates per treatment variant. Culture efficiency was calculated from 15–35 explants in every variant. The treatments are called 1D for one-day exposure to 2,4-D, 2D for two days of exposure, etc.

HISTOLOGICAL ANALYSIS

The material for sectioning was embedded in Technovit 7100 (2-hydroxyethyl-metacrylate) (Heraeus Kulzer). The cultured fragments of hypocotyls and cotyledons of all treatment variants were sampled after 1, 3, 5, 7, and 10 or 19 days of culture and fixed in glutaraldehyde for 24 h, then washed four times in phosphate buffer (PBS), dehydrated in a graded ethanol series for 15 min at each concentration (10%, 30%, 50%, 70%, 96%) and kept overnight in absolute ethanol. The next day the samples were infiltrated in mixtures of absolute ethanol and Technovit for 1 h at each proportion (3:1, 1:1, 1:3 v/v) and stored for 12 h in pure Technovit. The resin was polymerized with the addition of hardener. The material was sectioned at 5 µm with a rotary microtome (Microm, Adamas Instrumenten), stained with toluidine blue, and mounted in Entellan (Merck).

Photographs of in vitro cultures were taken under a Zeiss Stemi SV 11 stereomicroscope equipped with an MC80 microphotographic attachment on Kodak film. Microscope sections were photographed with a Zeiss Axio Cam MRe digital camera using Zeiss Axio Vision 3.0 software.

TABLE 1. *Brassica napus* L. cv. Kana. Scheme of variants of experiment cultured on induction medium (IM) and hormone-free growth medium (GM)

Variant*	No. of days on IM	No. of days on GM
control	0	28
1D	1	27
3D	3	25
5D	5	23
7D	7	21
14D	14	14
21D	21	7
28D	28	0

*variants indicated by the number of days of exposure to auxin and the letter "D"

RESULTS

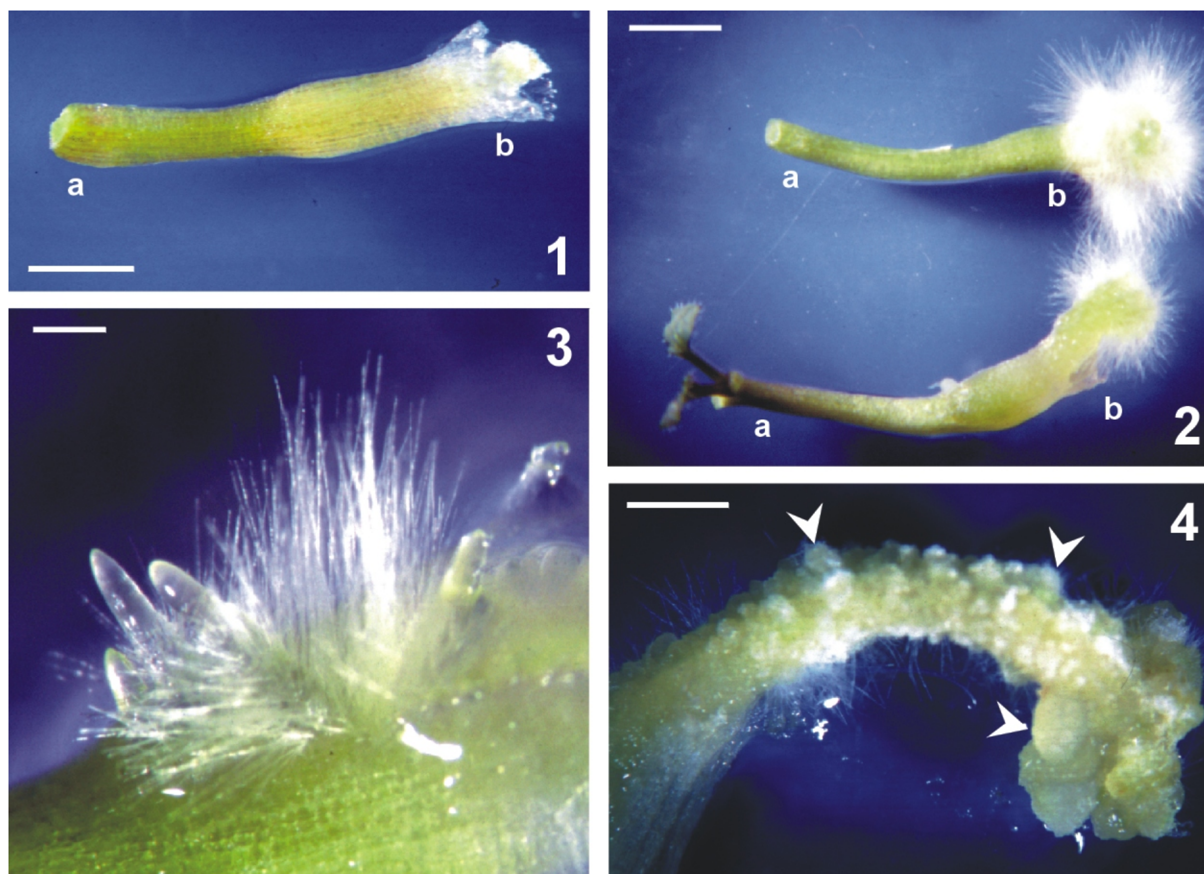
MORPHOLOGICAL OBSERVATIONS

Callus induction

Callus formation was restricted to the cut edges of explants or else started from them. Two morphological types of callus were noted after 28 days of culture: yellowish green, or yellow, granular callus, or white, watery, friable callus, both on hypocotyls and cotyledons. Longer time of exposure to 2,4-D (14D, 21D or 28D variants) influenced callus structure and resulted in the formation of yellow, compact callus (Fig. 4).

Hypocotyls. After 1 day of exposure to 2,4-D, differences in response between the apical and basal regions of the hypocotyl were distinct. After the next few days, epidermis sticking out from explants near basal cut edges was observed prior to callus formation (Fig. 1). Culture on 2,4-D-supplemented medium for 1–14 days resulted in the most abundant callus production. On explants cultured on hormone-free medium (control), callus was of very poor quantity, but callogenesis occurred on all explants (Tab. 2).

Cotyledons. Callus was induced on the 1D explants at ~54% efficiency. Longer periods of 2,4-D



Figs. 1, 2. Hypocotyls of 5D variant after 5 (**Fig. 1**) and 7 (**Fig. 2**) days of culture, showing differences between the apical (**a**) and basal (**b**) regions; intensive callus and trichome formation on the basal region, and shoot regeneration on the apical region (**Fig. 2**). Bars = 2 mm. **Fig. 3.** Hypocotyl of 3D variant after 10 days of culture, showing short roots with long root hairs and trichomes visible on part of the basal region. Bar = 0.5 mm. **Fig. 4.** Basal region of hypocotyl of 14D variant after 28 days of culture, showing abundant callus formation with nodular structures (arrowheads), very few trichomes, and no roots. Bar = 2 mm.

exposure induced callogenesis on 88.7–100% of the cotyledons, but on 28D explants the callus was of poor quantity. On control explants, callus formed sporadically (8% of explants) (Tab. 3).

Morphogenic response of explants

Cotyledon explants in the control, 1D and 3D variants enlarged significantly (~10-fold increase), and 2–4-fold in the other treatment.

Root formation was observed on hypocotyls and cotyledons, but shoots developed only on hypocotyls (Tabs. 2, 3). Most frequently, regeneration was obtained indirectly via callus. The duration of exposure to 2,4-D strongly affected root formation. Short treatment with 2,4-D (1D, 3D) was optimal for rhizogenesis; 100% of the 1D hypocotyls and 98% of the 3D variant developed roots (Tab. 2). Similarly, rhizogenesis on cotyledons was highly efficient in the 1D (80%) and 3D (54%) treatments (Tab. 3).

Root hairs and trichomes observed on 1D and 3D variants were numerous, measuring ~5 mm in length (Fig. 3). Longer exposure of explants (14D, 21D, 28D) resulted in inhibition of rhizogenesis on hypocotyls (Fig. 4, Tab. 2) and cotyledons (Tab. 3).

When explants were cultured on hormone-free medium (control), the frequency of explants producing roots varied depending on the explant type, reaching 80% for hypocotyls and 24% for cotyledons.

Adventitious shoot formation was lower than rhizogenesis. Shoots were observed exclusively on hypocotyls (Fig. 2). The best responses were from 3D variants (14% of explants formed shoots) (Tab. 2).

Long exposure to 2,4-D (28D) inhibited organogenesis. One shoot was formed on one hypocotyl; the rest of the explants, both cotyledons and hypocotyls, did not develop shoots or roots. Shoots and roots usually formed 3–4 days after transfer to GM medium.

Observations of organogenesis and callus formation indicated that the basal region of the hypocotyl is

TABLE 2. *Brassica napus* L. cv. Kana. Influence of 2,4-D on morphogenetic response of hypocotyls cultured 28 days (%)

Variant	No. of explants	No. of explants with callus	No. of explants with shoots	No. of explants with roots
Control	50	50 (100) +	1 (2.0)	40 (80.0)
1D	35	35 (100) +++	1 (2.8)	35 (100)
3D	50	50 (100) +++	7 (14.0)	49 (98.0)
5D	50	49 (98.0) ++	2 (4)	11 (22.0)
7D	75	69 (92.0) +++	1 (1.3)	16 (21.3)
14D	70	70 (100) +++	2 (2.8)	0
21D	50	50 (100) ++	0	0
28D	100	97 (97.0) ++	1 (1.0)	0

Quantity of callus: + poor; ++ medium; +++ abundant

TABLE 3. *Brassica napus* L. cv. Kana. Influence of 2,4-D on morphogenetic response of cotyledons cultured 28 days (%)

Variant	No. of explants	No. of explants with callus	No. of explants with shoots	No. of explants with roots
Control	50	4 (8.0) +	0	12 (24.0)
1D	35	19 (54.2) ++	0	28 (80.0)
3D	50	47 (94.0) ++	0	27 (54.0)
5D	40	40 (100) ++	0	17 (42.5)
7D	70	70 (100) +++	0	1 (1.4)
14D	80	71 (88.75) +++	0	0
21D	85	85 (100) ++	0	0
28D	115	108 (93.9) +	0	0

Quantity of callus: + poor; + + medium; + + + abundant

more suitable for callus and root formation; the apical region is able to form shoots.

HISTOLOGICAL STUDIES

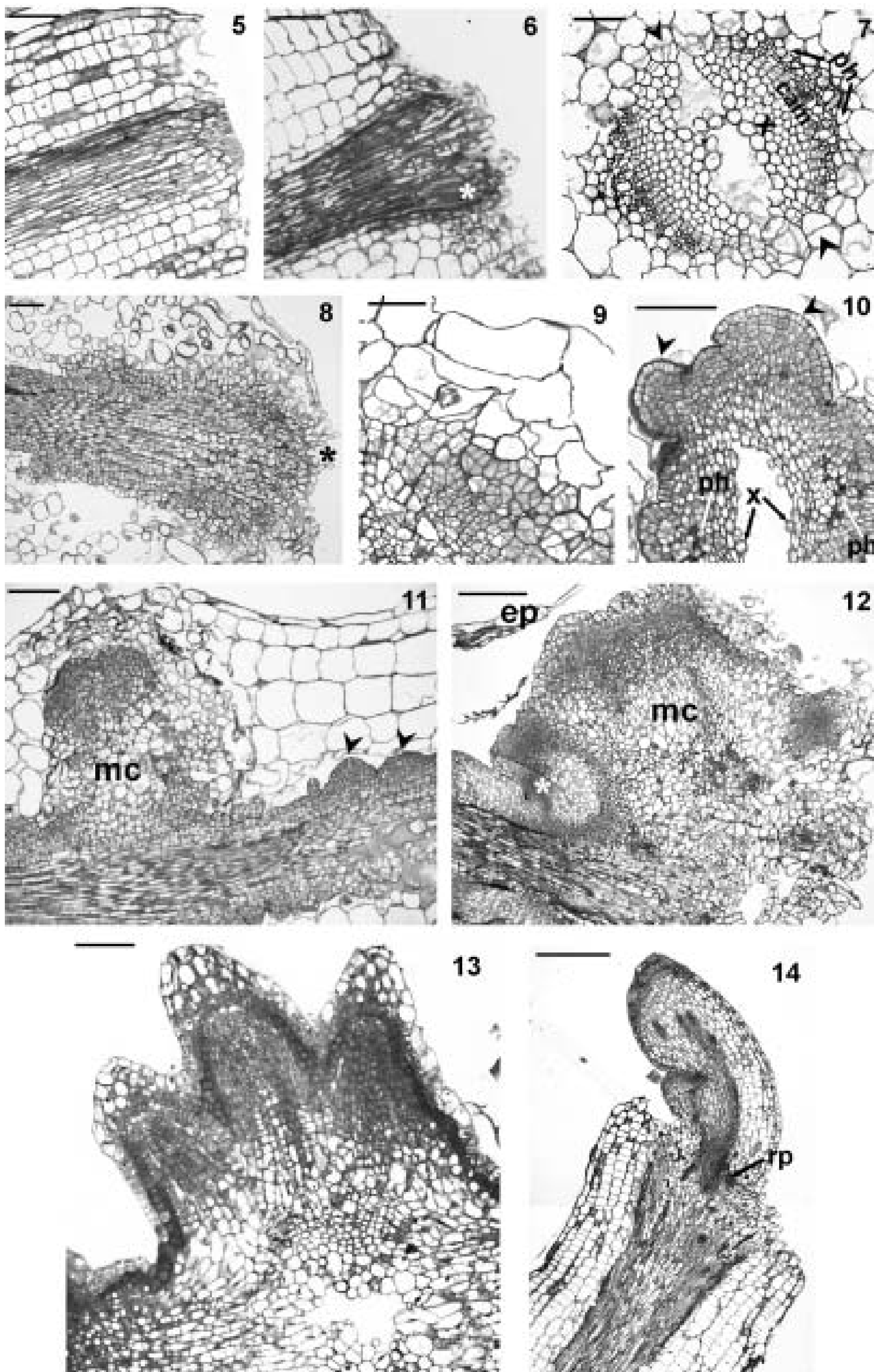
A difference in response between the two regions of the hypocotyl explants was observed after only 1 day of exposure to 2,4-D: the basal region was more swollen than the apical region. Sections of the 1D variant revealed enlarged cells in the cortex layer and stele as compared with control material cultured 1 day on hormone-free medium (Figs. 5, 6). After 5 days, the epidermis of the 3D variant protruded from the explants near basal cut edges. Cell proliferation was noted in the stele, especially around the vascular bundles and in the inner cortex (cells of endodermis) (Fig. 7). Cortex cells were weakly attached each other, and tissue was outgrown from the explant (Fig. 8); this was more clearly visible in sections after the following days of culture (Fig. 12). Parenchyma cells were large and vacuolated, while the cells of meristematic sites were small, with dense cytoplasm and without intercellular spaces (Fig. 9). In the region of the stele, many meristematic sites were noted (Fig. 10). Their proliferation resulted in callus induction (Figs. 11, 12) or rhizogenesis (Fig. 13). Some unorganized xylem elements (e.g., vessels with scalariform wall thickening) were observed in the calli masses.

Changes in cell organization inside the stele were observed on hypocotyls of the 3D variant after 5 (Fig. 7), 7 (Fig. 10) and 10 (Fig. 13) days of culture. Sections of younger explants revealed vascular bundles with phloem, procambium and xylem clearly visible. Proliferation of cells started between vascular bundles (next to phloem and procambium), but cells divided also in the cortex layer (Fig. 7). A few days later, vascular bundle elements were poorly recognizable (Fig. 10). Bulbs containing meristematic cells were located above remnants of vascular bundles. Cells of the cortex layer were weakly attached above the proliferation area. The growth of meristematic centers could result in callus formation (Fig. 11) or direct rhizogenesis (Fig. 13). Shoots with a visible root pole formed on the apical region of the hypocotyl (Fig. 14).

DISCUSSION

Intra-varietal variability of the organogenetic and callogenic response to plant growth regulators may be as great as the variability between *Brassica* species (Dietert et al., 1982), so the results obtained in one variety cannot be extended to the species. In our experiments we observed root formation only on explants cultured on hormone-free medium or briefly exposed to 2,4-D for

Figs. 5, 6. Longitudinal sections of basal region of hypocotyl. Hypocotyl of control (**Fig. 5**) and 1D variant (**Fig. 6**) after 1 day of culture, showing enlarged cells, especially in the stele region (asterisk). Bars = 100 µm. **Fig. 7.** Transversal section of hypocotyl of 3D variant after 5 days of culture, showing induction of cell division (arrowheads) at sites between vascular bundles (cam – procambium; ph – phloem; x – xylem). Bar = 50 µm. **Fig. 8.** Longitudinal section of basal region of hypocotyl of 3D variant after 5 days of culture, showing parenchyma cells of cortex layer weakly attached to each other and trichomes (asterisk) at end of vascular bundle. Bar = 200 µm. **Figs. 9, 10.** Transversal sections of hypocotyls of 3D variant after 5 days (**Fig. 9**) and 7 days (**Fig. 10**) of culture, showing meristematic sites (arrowheads) above remnants of vascular bundles (ph – phloem; x – xylem in Fig. 10). Bars = 50 µm in Fig. 9 and 100 µm in Fig. 10. **Figs. 11, 12.** Longitudinal sections of hypocotyls of 3D variant after 7 days of culture, showing meristematic sites (arrowheads) above stele, mass of callus tissue (mc) growing out of cortex and epidermis (ep), and organogenesis (asterisk). Bars = 200 µm in Fig. 11 and 0.5 mm in Fig. 12. **Fig. 13.** Transversal section of hypocotyl of 3D variant after 10 days of culture, showing direct formation of roots. Bar = 200 µm. **Fig. 14.** Longitudinal section of hypocotyl of 3D variant after 19 days of culture showing direct regeneration of shoot from apical region, and root pole (rp) below leafy structures. Bar = 0.5 mm.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up to 7 days. Callus formation was highly stimulated by exposure to 2,4-D, but long exposure resulted in poor callus growth. Shoot formation was observed exclusively on hypocotyl explants.

Callus initially formed mainly along the cut edges of cotyledons and hypocotyls, but the cell proliferation reached deeper parts of the explant in the following days. Similarly, Ullah et al. (2004) reported that on *Brassica napus* cv. Rainbow explants, callus proliferation started from the cut ends of the hypocotyl. Hofmann et al. (2004) reported different results in soybean: 2,4-D induced callus production over the entire surface of the cotyledon. The type and quantity of callus and callogenesis efficiency depended on the duration of exposure to 2,4-D and on the type of explant. In our experiments the highest quantity of callus was formed on hypocotyls and cotyledons after short induction on IM. Longer exposure to auxin caused poor formation of compact calli, with many necrotic sites. No roots and only one shoot formed on explants after 28-day exposure to auxin. Bogunia and Przywara (2000) obtained similar results in *Brassica napus* cv. Evita: media with 2,4-D as the sole growth regulator influenced callus proliferation but no regeneration; on hormone-free media, callus formed very sporadically. In other *Brassica* species, 2,4-D was shown to be necessary for callus formation on young hypocotyl explants (Dietert et al., 1982).

Our data confirmed previous results on *Brassica napus* var. *oleifera* cv. Skrzeszowicki (Rogozińska and Drozdowska, 1980), concerning the effect of 2,4-D on callus growth, and its inhibitory effect on root formation. Similarly, in *Daucus carota* (Jimenez and Bangerth, 2001) the presence of 2,4-D in the culture medium promoted callus induction and proliferation, while culture in the absence of 2,4-D stimulated root development at one end of the hypocotyl segments.

In our studies, shoot formation was observed, but only on hypocotyls and with low efficiency, in accordance with Khan et al.'s (2002) results. On hypocotyl explants of *Brassica napus* cv. Oscar cultured on 2,4-D-supplemented media, shoot regeneration was induced sporadically. Klimaszewska and Keller (1985) reported that continuous culture on media supplemented with 2,4-D did not produce any shoot formation. The differences between their results and ours may be attributable to differences between genotypes, but it is also possible that transferring hypocotyls to hormone-free medium is critical to shoot formation. Results obtained by Zheng and Konzak (1999) in *Triticum aestivum* clearly showed that the continuous presence of a 2,4-D concentration that satisfied callus induction inhibited further development and subsequent plant regeneration. Switching the culture to a lower 2,4-D concentration in the medium is needed for efficient regeneration of plants from calli. The continuous presence of 2–4 mg/l 2,4-D during induction beyond the critical point at which genes encoding products for plant regeneration are expressed is detrimental to the normal development of calli and may cause the loss of their regeneration capacity. In other species, shoot formation was observed when 2,4-D was accompanied by other growth regulators. Such observations were described in, for example, *Pleione formosana*: medium with 2,4-D induced callus only, while combining 2,4-D with TDZ (thidiazuron) produced shoot buds (Lu, 2004). Sairam et al. (2003) demonstrated callus induction and shoot bud differentiation from excised ends of soybean cotyledons after culture on media containing 2,4-D and benzyladenine.

Many studies have confirmed that the use of 2,4-D is critical to induction of somatic embryogenesis (e.g., Choi et al., 1998; Wang and Wei, 2004). However, in some species and genotypes, continuous long-term auxin treatment can inhibit somatic embryogenesis completely (Anzidei et al., 2000). The 2,4-D concentration used in our experiment (2 mg/l) was optimal for induction of embryogenic callus derived from immature embryos of wheat (Zhang et al., 2000). In contrast, the same concentration of 2,4-D in medium for culture of maize callus yielded only rhizogenic callus and root formation (Emons et al., 1993). In the present experiment, no somatic embryo formation was obtained. Since induction of somatic embryogenesis requires long-term culture – for example, 2, 4 and 6 months in callus of *Foeniculum vulgare* (Anzidei et al., 2000) – 28 days of culture may be too short for somatic embryos to develop on *B. napus* explants.

Culture with 3 and 5 days of exposure to 2,4-D induced the most shoots (14% and 4%, respectively) and relatively high root production on hypocotyls (98.0% and 22.0%, respectively). The low efficiency of shoot induction on cultured tissue in the present experiment may be connected with the reaction of *Brassica napus* cv. Kana to the composition of the culture medium. It is known that culture conditions as well as the plant genotype have a significant impact on shoot regeneration frequency (Hu et al., 1999). In *Brassica napus* ssp. *oleifera* cv. Westar, shoot organogenesis occurred only in the presence of NAA in the culture medium (Klimaszewska and Keller, 1985), and on *Brassica napus* cv. Oscar explants the highest frequency of shoot regeneration was achieved on medium with 2 mg/l BAP and 0.5 mg/l IAA (Khan et al., 2002).

Histological observations revealed that cell proliferation started between and above the vascular bundles of hypocotyls. Meristematic sites seemed to originate from cells of the pericycle or endodermis. It is well known that the pericycle of roots has meristematic potential. Morphological observations showed that root formation was more efficient on the basal region of the hypocotyl. In this part of the explant, the pericycle could more easily switch to the organogenesis pathway. Similar cell divisions near vascular bundles leading to

the formation of meristematic centers were observed in *Gentiana pneumonanthe* leaf explants (Bach and Pawłowska, 2003).

Our observations indicated that the response of hypocotyl explants to hormone-free media and to media supplemented with 2,4-D is highly polarized. Root, trichome and callus formation started mainly from the basal region of the hypocotyl, but the few shoots obtained in the experiment appeared only on the apical region of the hypocotyl. Similar observations were made in bean (Angelini and Allavena, 1989). This indicates that auxin significantly increases the competition response of the two hypocotyl regions.

Plant explants in culture are usually inoculated on media containing stable concentrations of growth regulators, and then are moved to new media after a few weeks. The present study confirmed that the response of explants cultured on media containing 2,4-D depends not only on the concentration of the phytohormone but also, and to a great extent, on the duration of exposure to growth regulator. The meristematic sites probably could develop in roots or shoots only after transfer to hormone-free medium. Continuous long-term auxin treatment inhibited the morphogenic response of the explants, a phenomenon which should be considered when experiments are planned.

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It is a great honor for us to participate in this special volume dedicated to the memory of our teacher, Professor Lesław Przywara.

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