Plant Cell Tissue Organ Culture 4 (1985) 183–197

High frequency plant regeneration from thin cell layer explants of *Brassica napus*

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(Received August 31, 1984; in revised form January 30, 1985; accepted January 31, 1985)

Key words: *Brassica napus*, thin cell layers, organogenesis, plant regeneration, rapeseed, explant

Abstract. Explants composed of the epidermis and 4–9 layers of subepidermal cells were excised from internodes of *Brassica napus* L. ssp. *oleifera* cv. Westar and cultured on modified Murashige and Skoog (MS) medium. The three or four terminal internodes excised from plants at an early stage (before any flower buds had opened) were shown to be the best explant source. Both cytokinin and auxin were required for induction of shoot organogenesis. Of six auxins tested, only naphthaleneacetic acid (NAA) was effective in shoot bud initiation. All four cytokinins tested (when associated with 0.5 mgl^{-1} NAA) promoted organogenesis, but at differing frequencies. The highest shoot induction frequency was obtained at 10–15 mgl^{-1} benzyladenine (BA). The organogenic response was strongly affected by the nitrogen content of the medium. The best response was observed when NO_3^- was the sole nitrogen source (supplied as KNO_3) in the range 30–90 mM. Sucrose and glucose were equally supportive in shoot regeneration with the optimal levels at 0.12 M and 0.15 M, respectively. Shoots were rooted on medium free of growth regulators and mature plants were grown in the greenhouse. Plants were also recovered from leafy structures which differed morphologically and histologically from shoot buds.

Introduction

The thin cell layer system was first described by Tran Thanh Van and co-workers [33, 34] to study morphogenesis in *Nicotiana tabacum*. Explants excised from floral branches, composed of 3–6 layers of epidermal and subepidermal cells, were capable of either flower or shoot bud formation. The system was also applied in shoot regeneration of *Begonia rex* [4], *Torenia fournieri* [3], *Bryophyllum daigremontianum* [1] and *Psophocarpus tetragonolobus* [11].

Brassica napus (oilseed rape) has become the object of extensive tissue culture studies. Effective regeneration protocols would be desirable for maintenance of both haploid and self-incompatible lines. In addition, somaclonal variation occurring amongst in vitro regenerated plants could be a useful breeding tool [18]. To date, organogenesis has been achieved in a variety of *B. napus* explants including: seedling tissue [6, 26], floral parts

Contribution No. 763 Ottawa Research Station.

[23], stem internodes [14, 30, 31], leaves [7, 27, 31], and roots [10, 19]. Embryogenesis has been reported in hypocotyl tissues [17, 22, 32], stem internodes [30], anther and pollen cultures [16, 20] and protoplast-derived cell colonies [21]. In this paper we describe factors influencing morphogenesis in thin cell layer explants isolated from internodes of *B. napus* stems.

Materials and methods

Seeds of *Brassica napus* L. ssp. oleifera cv. Westar were obtained from Dr. R.K. Downey, Research Station, Agriculture Canada, Saskatoon. They were sown in 12 cm pots containing soil : peat : sand mixture (1 : 2 : 1) and seedlings were established in the greenhouse. Ten-day-old plantlets were transferred to a growth chamber with day : night temperature of 20 : 15 °C, 16 h photoperiod and light intensity of 400 μE m^{-2} sec^{-1} derived from cool white fluorescent tubes (type: F96/T12/VHO) and incandescent (45W) bulbs producing 84 and 16% of the intensity, respectively. Relative humidity was maintained at 70%. The plants were watered daily and fed with 20 : 20 : 20 (N : P : K) liquid nutrient and Hoagland's solution once per week.

Three or four terminal internodes from flowering plants (with a few flower buds opened, unless otherwise indicated) were removed and surface sterilized in 70% v/v ethanol for 1 min, then in 2% w/v sodium hypochlorite for 20 min followed by 3 rinses in sterile distilled water. Thin cell layer strips composed of epidermis plus 4–9 layers of subepidermal cells (Figure 6) were isolated longitudinally from the internodes using Beaver 75 micro-sharp blades (Waltham, MA, USA). These strips taken randomly from the internodes were cut into pieces approximately 2–3 mm wide and 8 mm long and placed with subepidermal surface in contact with the medium. Five explants per dish were cultured in 60 x 20 mm plastic petri dishes containing 16 ml ± 0.5 ml of medium. The basic medium consisted of macro- and microelements according to Murashige and Skoog [24] abbreviated as MS medium (and vitamins according to Gamborg et al. [8]) with 40 mgl^{-1} of sequestrene Fe-330 (Ciba-Geigy) as a source of iron. Unless otherwise indicated, all media contained 0.5 mgl^{-1} naphthaleneacetic acid (NAA), 5 mgl^{-1} benzyladenine (BA), 3% w/v (0.09 M) sucrose and 0.8% w/v Difco-Bacto agar. The pH was adjusted to 5.8 with 0.1 N KOH prior to autoclaving. Except for experiments on light conditions, cultures were maintained under continuous fluorescent light (approximately 70 μE m^{-2} sec^{-1}) at 25 °C. The number of explants with shoot buds and roots was scored after 1 and 2 months of culture. The percentage of regenerating explants was calculated based on 30 explants per treatment from each of at least two replications.

Well developed shoots were separated and transferred to MS medium lacking growth regulators in order to induce rooting. The small plantlets were transferred to peat pellets and placed in a mist chamber for 10 days prior to potting and maintenance in a greenhouse.

For histological observations, explant pieces were fixed in formalin-acetic acid-alcohol (FAA) for 24 hours. The fixed tissues were dehydrated in t-butanol series, embedded in paraffin (Paraplast T-M 56–57 °C) and sectioned at 7 μ. They were subsequently stained with safranin and fast-green, and mounted in Canada balsam. For preparation of 1–2 μ sections the tissues were dehydrated in ethanol-propylene oxide series embedded in Spurr [29] resin, cut with an ultramicrotome and stained with toluidine blue.

Results

Thin cell layer explants of *B. napus* enlarged significantly after 4–5 days of culture. The growth was greater in explants isolated from terminal internodes than in those taken from the basal regions. After 7–10 days a small amount of light-green callus proliferation was observed on the subepidermal surface. Shoot buds could be detected after 12–15 days, usually on one end of the explants, however, their formation around the edges was observed occasionally (Figure 1). Roots were formed on both regenerating and non-regenerating shoot bud explants. After 3–4 weeks in some treatments (see below), dark-green or purplish meristematic centers were observed. They later developed into leafy green structures (Figure 2). The prominent feature of these structures was the lack of elongated internodes and many of them were embryo-like in appearance. Both shoot buds and leafy structures could originate from the same explant. None of the parameters evaluated in this study influenced the frequency of appearance of these structures. Upon transferring to growth regulator-free MS medium the leafy structures developed into normal shoots.

All shoots were easily rooted on basal medium and could be transferred to the greenhouse (Figures 3 and 4) where they matured and set seed.

Effect of development stage of the donor plant

Plants at three developmental stages were used as a source of explants. These included plants that were 30 days old in which the inflorescence-bearing stem had not elongated (stage I), those that were 37–38 days old with elongated stems but no open flowers (stage II) and those 42–43 days old in which a few flower buds had opened (stage III). The highest frequencies of shoot and root organogenesis occurred in explants derived from stage II plants.

Stage II plants with at least 15 internodes were used in an experiment to determine the significance of their position on the stem on subsequent in vitro organogenesis. Twelve internodes (IV–XV) counting from the base upwards were used as explant sources. The highest frequencies of shoot regeneration were obtained from the uppermost internodes (Table 1). For subsequent experimentation, 3–4 terminal internodes were used.

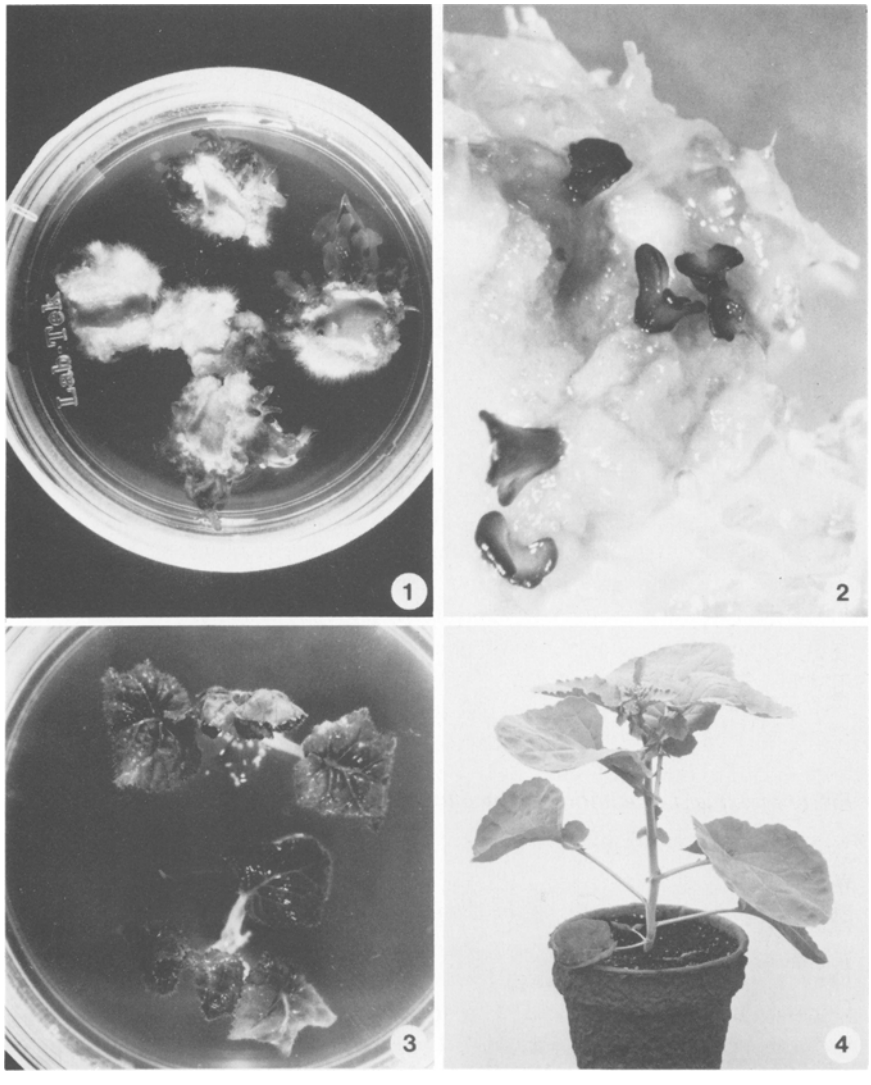

Figure 1. Shoot bud and root development from thin cell layer explants after 15 days of culture on MS + 0.5 mgl^{-1} NAA + 5 mgl^{-1} BA (1.4 ×).

Figure 2. Leafy structures emerging from callus after 4 weeks of culture on MS + 0.5 mgl^{-1} NAA + 5 mgl^{-1} BA (12 ×).

Figure 3. Regenerated shoots excised from callus and placed on rooting medium (1.9 ×).

Figure 4. Plant at early flowering stage established in the greenhouse (0.4 ×).

Table 1. Influence of source of explant (based on position on the stem) on shoot organogenesis in thin cell layers of *B. napus*

Explant source (internode)	Frequency of explants producing shoots (%)
IV–VI	42
VII–IX	55
X–XII	77
XIII–XV	87

Table 2. Effect of auxin type on in vitro response of thin cell layers of *B. napus*

Auxin*	Concentration (mgl^{-1})	Response
NAA	0.5	callus, shoots, roots
	2	callus, shoots, roots
2,4-D	0.5	callus
	2	explants enlarged
IBA	0.5	callus
	2	explants enlarged
IAA	0.5	explants enlarged
	2	callus
2,4,5-T	0.5	callus (dark green compact)
	2	callus (dark green compact)
Picloram	0.5	explants enlarged
	2	necrosis
None	–	necrosis

*BA concentration = 5 mgl^{-1}

Effect of explant orientation on medium

The orientation of explants strongly influenced their organogenic response. When the epidermis was in contact with the medium the frequency of explants regenerating shoots and roots decreased from 40 to 13%.

Effect of growth regulators

Organogenesis in cell layers was strongly dependent upon growth regulator type and concentrations. On growth regulator-free medium, the explants senesced within a few days. If either auxin or cytokinin was used alone, shoot regeneration was not observed. Six auxins [NAA, 2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), Picloram] applied at 0.5 or 2 mgl^{-1} were tested in combination with BA at 5 mgl^{-1}, but shoot formation occurred only in the presence of NAA (Table 2). Other auxins caused either explant enlargement and in some cases, callus production. Tests with a range of NAA concentrations revealed that the highest frequencies of organogenesis occurred at 0.5–1 mgl^{-1} (Table 3).

Table 3. Effect of NAA concentration on shoot regeneration in thin cell layers of *B. napus*

NAA conc.* (mgl^{-1})	Frequency of explants producing shoots (%)	No. of shoots per explant
0	0	0
0.1	16	1–5
0.5	61	5–10**
1	63	5–10**
2	55	5–10
4	58	5–10
6	17	1–5
8	10	1–5
10	0	0

*BA conc. = 5 mgl^{-1}
**Leafy structures observed

Table 4. Effect of cytokinin type on shoot regeneration in thin cell layers of *B. napus*

Cytokinin type*	Conc. (mgl^{-1})	Frequency of explants producing shoots (%)
BA	1	33
	5	47
BA riboside	1	33
	5	33
Kinetin	1	20
	5	33
2iP	1	10
	5	30
Zeatin	1	17
	5	30
None	–	0

*NAA concentration = 0.5 mgl^{-1}

Table 5. Effect of BA concentration on shoot regeneration in thin cell layers of *B. napus*

BA conc.* (mgl^{-1})	Frequency of explants producing shoots (%)	No. of shoots per explant
0	0	0
0.1	40	5–10**
0.5	42	5–10**
1	44	5–10**
2	48	10**
5	50	>10**
10	67	>10**
15	67	>10**
20	53	>10**
25	58	>10**
30	52	>10**
35	28	10
40	8	1–5

*NAA conc. = 0.5 mgl^{-1}
**Leafy structures observed

Cytokinins at 1 and 5 mgl^{-1} were added to medium with NAA at the constant level of 0.5 mgl^{-1}. All cytokinins [BA, BA-riboside, kinetin, N^6 (Δ^2-isopentenyl) adenine (2iP), zeatin] induced shoot bud formation with BA being the most effective (Table 4). On cytokinin-free medium the cell layers enlarged slightly and occasionally produced a few roots. A range of BA concentrations was studied with maximal frequencies of shoot formation occurring at 10–15 mgl^{-1} (Table 5). Even concentrations as high as 30 mgl^{-1} caused only a slight decrease in shoot bud formation, however, the organogenic response was delayed (occurring after 4–5 weeks of culture). In the concentration range of 5–30 mgl^{-1}, there were always more than 10 shoot buds initiated per responding explant.

A range of gibberellic acid (GA_3) concentrations (0.1–10 mgl^{-1}) was tested in combination with 0.5 mgl^{-1} NAA and 5 mgl^{-1} BA. GA_3 did not stimulate shoot organogenesis and at concentrations greater than 0.1 mgl^{-1} it was inhibitory.

Effect of NO_3^-/NH_4^+ ratio

At a constant nitrogen level of 60 mM (the level in MS medium) variation in nitrate to ammonium ratio strongly affected the organogenic response (Table 6). No shoot buds developed when NH_4^+ was the sole nitrogen source. There was a gradual increase in the shoot formation frequency as NH_4^+ concentration was decreased with maximal frequency observed when NO_3^- was the sole nitrogen source. The results obtained on control MS medium with NO_3^-/NH_4^+ ratio of 2/1 indicated that there was little difference in response when the same ratio was held constant but other NH_4^+ sources were used (Table 6). Various nitrogen levels were tested using KNO_3 as the only source (Table 7). The highest shoot induction frequency was observed in the range of 30–90 mM KNO_3.

Effect of carbohydrates

Both sucrose and glucose supported shoot bud formation across a wide range of concentrations (Table 8 and 9). The optimal concentration of sucrose was 0.12 M (4%) and that of glucose 0.15 M (3%). A significant reduction in number of regenerating explants was noted when concentrations greater than 0.23 M sucrose and 0.3 M glucose were used.

Effect of agar concentration

Explants floated on liquid medium failed to produce any shoots. Even a very low (0.2%) agar concentration markedly increased shoot regeneration frequency with the optimal response occurring in the range of 0.8–1.2%.

Effect of light

The effect of light conditions was studied by maintaining the explants in either darkness or under continuous fluorescent light or 16 h photoperiod.

Table 6. Effect of nitrate: ammonium ratio on shoot regeneration in thin cell layers of *B. napus*

NO_3^-:NH_4^+	NO_3^- conc. (mM)	NH_4^+ conc. (mM)	Frequency of explants producing shoots (%)	
			KNO_3 + NH_4^+ citrate	KNO_3 + NH_4Cl
–	0	60	0	0
1:16	3.5	56.5	0	0
1:4	12	48	0	0
1:1	30	30	2	56
2:1	40	20	44 (50)*	53 (54)*
4:1	48	12	52	60
16:1	56.5	3.5	68	67
–	60	0	76	70

*Control = modified MS medium with 20.6 mM NH_4NO_3 and 18.8 mM KNO_3

Table 7. Effect of KNO_3 concentration on shoot regeneration in thin cell layers of *B. napus*

KNO_3 conc. (mM)	Frequency of explants producing shoots (%)
30	77
60	80
90	83
120	60
150	30
Control*	47

*Modified MS medium with 20.6 mM NH_4NO_3 and 18.8 mM KNO_3

Table 8. Effect of sucrose concentration on shoot regeneration in thin cell layers of *B. napus*

Sucrose conc. M	% (w/v)	Frequency of explants producing shoots (%)
0.003	0.1	0
0.01	0.5	16
0.03	1	45
0.06	2	50*
0.09	3	53*
0.12	4	63*
0.17	6	43*
0.23	8	20
0.29	10	7
0.4	14	0

*Leafy structures observed

The highest freqency of shoot bud induction was obtained under continuous light (54%) with a reduced level under a photoperiod (26%) and the lowest induction occurring in darkness (7%).

Effect of explant preculture on induction medium

Explants were cultured for various periods of time on shoot induction medium prior to transfer to basal medium free of growth regulators. Explants

Table 9. Effect of glucose concentration on shoot regeneration in thin cell layers of *B. napus*

Glucose conc. M	% (w/v)	Frequency of explants producing shoots (%)
0.005	0.1	0
0.025	0.5	20
0.05	1	27
0.1	2	46*
0.15	3	50*
0.2	4	42*
0.3	6	37*
0.4	8	5*
0.5	10	0

*Leafy structures observed

Table 10. Effect of preculture on induction medium on morphogenesis in thin cell layers of *B. napus*

Length of preculture* (d)	Frequency of explants producing shoots (%)	Roots (%)
0	0	0
1	0	6
2	0	14
3	20	10
4	22	30
6	20	25
10	20	20
Continuous	35	40

*Explants cultured in the presence of 0.5 mgl^{-1} NAA and 5 mgl^{-1} BA prior to transfer to growth regulator-free medium

had to be cultured on induction medium for at least three days in order to achieve shoot bud formation (Table 10). In contrast, one day preculture on medium with growth regulators was sufficient to induce low frequencies of root formation. However, continuous maintenance of explants on medium with growth regulators was superior for both shoot and root organogenesis.

Histology

Histological sections of primary thin cell layer explants were variable in regard to number of component cell layers within an explant as well as amongst explants. The number of subepidermal layers, which included collenchyma and parenchyma, varied from three to nine but never included vascular tissue (compare Figure 5 with Figure 6). The first periclinal divisions occurred in lower cell layers (Figure 7). Cells in the other layers subsequently underwent divisions leading to callus proliferation. Centers of meristematic activity could be detected in callus tissue after 5–7 days (Figures 8 and 9). Differentiation of strands of procambial cells (Figure 10) as well as tracheids was also observed. Shoot meristems differentiated on the callus surface

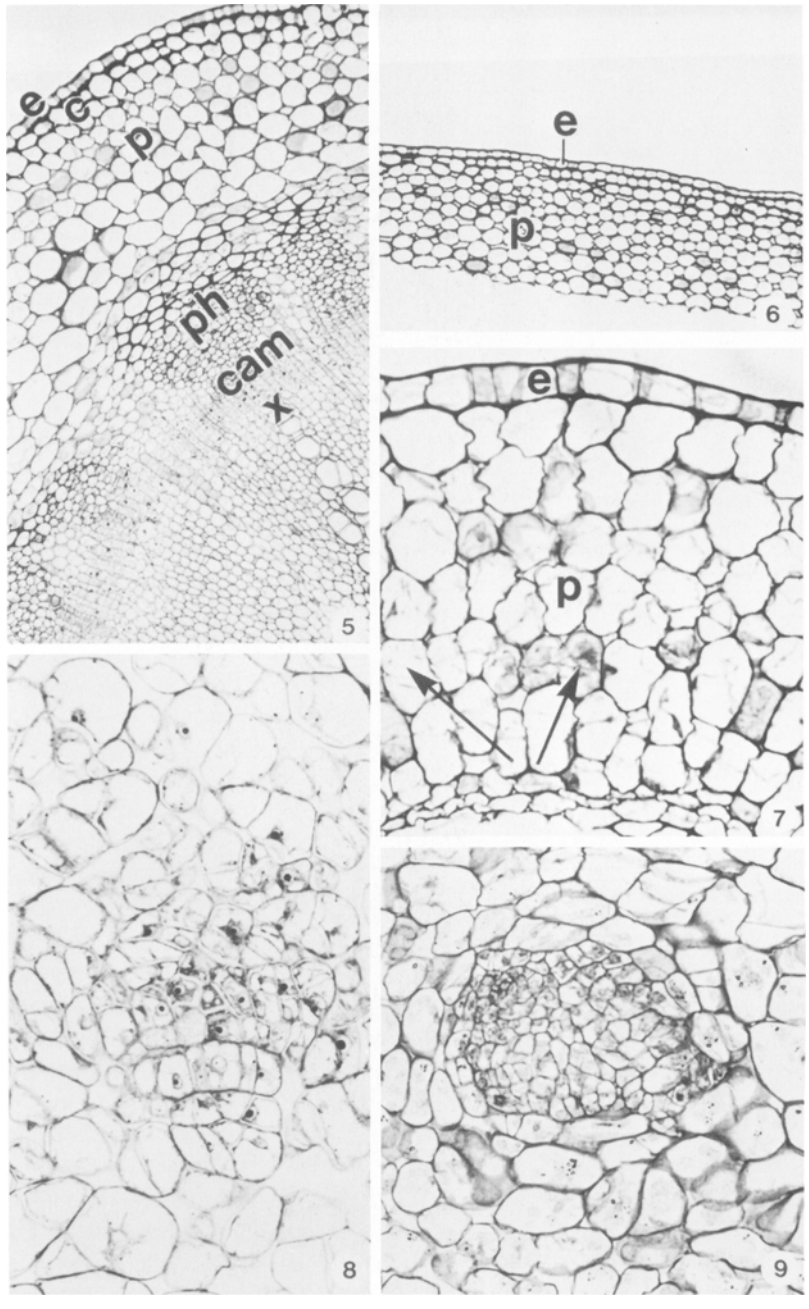

Figure 5. Fragment of transverse section of *B. napus* cv. Westar stem (63 X).

Figure 6. Fragment of transverse section of *B. napus* cv. Westar freshly isolated explant (25 X).

Figure 7. Frangment of transverse section of thin cell layer explant after 3 days of culture (160 X). Arrows indicate periclinal divisions.

An early (Figure 8) and an advanced (Figure 9) stage of meristematic center (160 X).

e = epidermis; c = collenchyma; p = parenchyma; ph = phloem; cam = cambium; x = xylem; sm = shoot meristem; 1p = leaf primordium; pcam = procambiun; rp = root pole, 1 = leaf.

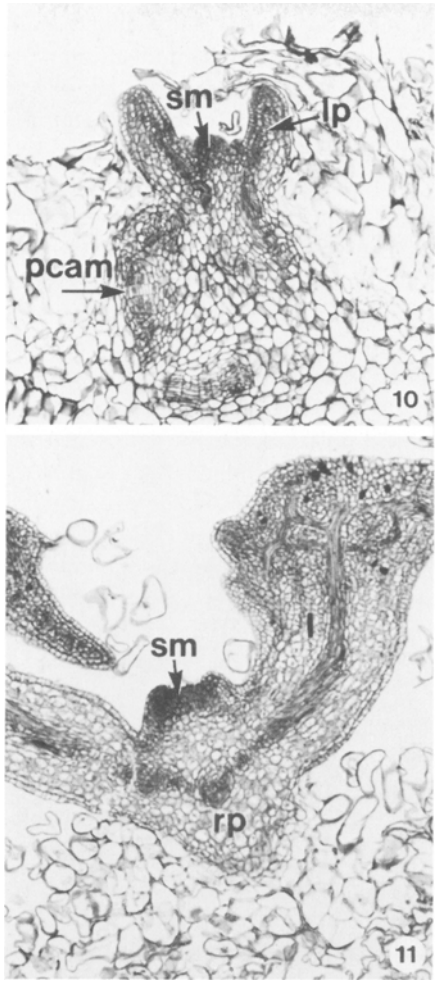

Figure 10. Longitudinal section of shoot bud (25 ×).

Figure 11. Longitudinal section of leafy structure (25 ×).

(Figure 10). Roots which developed from callus did not have any vasuclar connection with shoot primordia. Sections of leafy structures exhibited a precociously developed first pair of leaves and a very weakly organized root pole embedded in the surrounding tissue (Figure 11).

Discussion

Induction of shoot organogenesis in this study was dependent on a specific auxin-cytokinin interaction. Out of six auxins tested in the presence of

cytokinin, shoot organogenesis occurred only in the presence of NAA. This degree of auxin specificity is somewhat unusual but not unique. For example, NAA was specifically required for induction of embryogenesis in *Solanum melongena* [9] Thin cell layer explants apparently differed from other types of explants in their requirement for auxin. Kartha et al. [14] and Stringam [30, 31] were able to regenerate shoots from internode segments of either diploid or haploid *B. napus* on media using BA alone at 1 mgl^{-1}. In those studies, higher concentrations of BA inhibited organogenesis. Thin cell layer explants required much higher BA concentrations (10–15 mgl^{-1}) for maximal shoot induction. This may be related to explant sensitivity or the original content of endogenous growth regulators. Thin cell layers apparently have little carry-over of endogenous regulatory substances compared to larger complex explants [12]. Kartha et al. [14] also demonstrated lack of shoot formation on internode segments of *B. napus* when NAA was the sole growth regulator, but there was no auxin-specific effect in the presence of BA. In contrast, Grant and Harney [10] found NAA essentially inhibitory for shoot differentiation from root pieces of *B. napus* ssp. napobrassica, whereas IAA was stimulatory. Other workers have reported that the combination of NAA and BA was the most effective for shoot induction in *B. napus* cotyledons [26] and leaf discs [7]. In the latter case, maximal shoot differentiation occurred when 10 mgl^{-1} NAA with 10 mgl^{-1} BA was applied, while in cell layer explants such a high auxin concentration completely suppressed the organogenic response (Table 3).

GA_3 was not stimulatory for shoot bud regeneration in cell layers of *B. napus* over a wide range of concentrations tested. These results differ from those obtained by others who found GA_3 to be essential for shoot regeneration in leaf callus of rapeseed [27, 31] as well as in callus derived from mesophyll protoplasts [15, 25].

Shoot organogenesis in thin cell layers of *B. napus* was strongly affected by NO_3^-/NH_4^+ ratio and total nitrogen levels with the highest frequencies occurring on medium with NO_3^- as the sole nitrogen source. High NO_3^-/NH_4^+ ratios were also more favorable for shoot bud regeneration in cultured *B. napus* flower buds [23]. In African violet subepidermal tissue isolated from petioles, shoot organogenesis occurred on B_5 but not on MS medium leading the authors to conclude that the high NH_4^+ level in MS medium inhibited shoot induction [2].

Both sucrose and glucose were equally supportive for organogenesis in thin cell layer explants of *B. napus* with optimal concentrations at 0.12 M (4%) and 0.15 M (3%) respectively. Sucrose levels greater than 0.23 M (8%) and glucose levels greater than 0.3 M (6%) were inhibitory. These observations differed from those of Dunwell [7] who reported that shoot organogenesis in *B. campestris* leaf disks was inhibited if sucrose concentration was greater than 4%.

Maximal shoot regeneration frequency was obtained at agar concentrations

between 0.8–1.2%. Higher concentrations were inhibitory which probably was due to decreased availability of water. According to Debergh [5], Difco-Bacto agar is a source of potentially beneficial impurities and this fact might explain a significant difference in thin cell layer explant response to liquid and solidified media. The reduction in shoot formation frequency in liquid medium as compared with agar has also been reported for cultured *B. napus* root segments [19].

Shoot bud formation in cell layers of *B. napus* occurred at the highest frequency in cultures kept continuously in light. In darkness only sporadic shoot regeneration was observed. Dietert et al. [6] maintained hypocotyl cultures of *B. napus* in darkness to induce callus production but in order to induce organogenesis the cultures were transferred to light. Other authors have applied photoperiod conditions to achieve organogenesis in various types of *B. napus* explants [7, 10, 14, 26, 30, 31], however, they did not generally evaluate the optimal light requirements.

Continuous maintenance of explants on induction medium was more effective than preculture for limited periods (Table 10). These results differ from those described by Hanh et al. [11] on winged bean thin cell layers in which optimal shoot bud induction occurred after 7 days of contact with the primary medium and culture for more than 15 days on this medium was inhibitory.

The capacity of thin cell layers to regenerate shoots was influenced by explant position on a stem as well as developmental stage of a donor plant with the highest frequencies of shoot proliferation occurring in explants taken from terminal internodes. Similar results have been reported for shoot regeneration in internode segments of *B. napus* [30] and *B. oleracea* [13].

A histological study of thin cell layer explants revealed that callus originated from subepidermal tissues. Epidermal cells cultured as monolayers did not undergo divisions (data not shown). Shoot and root meristems differentiated in callus tissue after 7- 10 days. According to Stringam [31], in *B. napus* stem and petiole explants the shoot primordia arose from the cambial regions. Our studies indicated that superficial stem tissues also had organogenic capacity. It is possible that the intertissue correlations (either hormonal or metabolic) in stem explants were responsible for the inhibition of organogenesis in superficial tissues [35]. Analysis of anatomical sections of leafy structures showed the presence of weakly differentiated 'root poles' thus it was possible to separate them from surrounding tissue. Although this feature is characteristic of embryo structures, other features like precocious leaf development with bluntly serrate edges and lack of recognizable hypocotyls led us to conclude that they were not true embryos. However, somatic embryogenesis in *B. napus* has been reported by Margara and Leydecker [23] and Stringam [31]. In the latter case, trumpet-shaped structures resembling embryos were observed in internode calli, however, these were not analyzed further. Secondary embryos have also been observed on hypocotyls of

anther-derived embryos [17, 32]. More recently, Singh and Chandra [28] have identified bipolar meristemoids resembling embryos in callus culture of *B. campestris.*

Thin cell layer explant cultures of *B. napus* ssp. oleifera cv. Westar have been shown to be an effective system for studying morphogenesis and could be a potential method for micropropagation. The system is capable of high shoot regeneration frequency and high number of shoots per explant. Furthermore, microbial contamination of explants was never a serious problem. It also provides a relatively uniform starting material which is presently being tested as a protoplast source.

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