ACTA SOCIETATIS
BOTANICORUM POLONIAE
Vol. 49, nr 1/2; 5—20
1980

Organogenesis and plant formation from cotyledon and callus culture of rape

JANINA H. ROGOZIŃSKA, LUCYNA DROZDOWSKA

Laboratory of Plant Physiology, Institute of Agriculture,
Technical-Agricultural Academy, Bernardyńska 6, 85-029 Bydgoszcz, Poland

(Received: June 15, 1979)

Abstract

Cotyledon explants of rape were excised from aseptically germinated seedlings and cultured during 2 weeks on Murashige and Skoog medium supplemented with auxins, cytokinins, auxin-cytokinin combinations and abscisic acid. Callus formation occurred on medium with 2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene-1-acetic acid (NAA), indole-3-acetic acid (IAA) and on their combinations with kinetin (K) or 6-benzylaminopurine (BAP). Regeneration of roots was achieved on media with NAA, IAA and indole-3-butyric acid (IBA) and on combinations of these auxins with cytokinins. The presence of 2,4-D in the medium, though it promoted compact callus growth, had an inhibitory effect on root formation. Callus derived from the cotyledons had somewhat different requirements for growth in subculture and the root formation ability diminished in the course of the culture. Lower ABA concentrations stimulated callus growth whereas higher concentrations inhibited it similary as in the case of cotyledons. Shoot buds regenerated from the cotyledons after ca. 3 weeks on media supplemented with NAA + BAP. The 9-week-old plantlets transferred to the soil developed into complete plants. The plants which underwent vernalization formed flowers and normal seeds.

INTRODUCTION

The ability to regeneration makes it possible to reproduce complete plants from isolated organs, tissues, cells and even protoplasts. To date, many plants of economic value have been successfully propagated by tissue culture techniques. Clonal multiplication of plants was applied to some plants from the *Brassicaceae* family, which are an important crop. These plants, however, often contain glucosinolates and erucic acid which lower their fodder value. Besides breeding, the tissue culture

technique may be of value in attempting to diminish their glucosinolates content (Rogozińska, Drozdowska, in press).

Some species from the *Brassicaceae* family, e.g. *Brassica napus* var. *napobrassica,* regenerated plants from storage roots and cotyledons (Drozdowska, Rogozińska, 1975, 1976). Also *Brassica juncea* cotyledons regenerated plants (Hui, Zee, 1978). However from rape seedlings, Afzalpurkar (1974) and Radwan (1975) obtained callus tissue only. Various other parts of rape were tested with varying success and it was possible to obtain adventitious buds from leaf blades, petioles and flower buds (Yie, 1978). Whole plant regeneration from internodal segments and isolated mesophyll protoplasts was achieved by Kartha et al. (1974a, b) and from shoot tips by Yie (1978).

In this paper the ability of plant regeneration from rape cotyledons, not tested hitherto, was established under well defined conditions. Further growth stages of the plantlets in the soil up to flower and seed formation were also followed.

MATERIAL AND METHODS

Brassica napus L. var. *oleifera* cv. 'Skrzeszowicki', with high glucosinolate content was chosen for experimental work. Seeds of this plant were surface-sterilized with 0,2% mercuric chloride for 5 min. and repeatedly washed in sterile water. The sterilized seeds were put in Petri dishes with moistened filter paper. After 40 ± 2 h imbibition the outside cotyledons were excised, cut in halves and placed on Murashige and Skoog (1962) medium (M.S.). The medium was supplemented with auxins, like naphthalene-1-acetic acid (NAA), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D) and cytokinins such as kinetin (K) and 6-benzylaminopurine (BAP). After 14 days the fresh and dry weight of the cotyledons was determined and its ability to callus formation and organogenetic differentiation recorded. In order to obtain bud formation the cotyledon culture on medium with NAA + BAP was prolonged to 9 weeks and the plantlets formed were planted into soil, transferred to the greenhouse and later to the field. Moreover, the influence of abscisic acid (ABA) was tested in combination with NAA and BAP, in order to check its eventual effects on growth and differentiation of rape.

In experiments on growth and differentiation of callus tissue, the callus derived from cotyledons was cultured on M.S. medium with 10 μM NAA + 10 μM BAP. It was transferred several times before use and, in these experiments, derived from the 6-8 subculture. Analogous plant growth substances as in the case of cotyledons were tested.

The cotyledons and tissue cultures were incubated under a photoperiod of 16-h light of ca. 1500 lux at 25°C ± 2.

RESULTS AND DISCUSSION

Effects of growth substances on the morphogenesis of isolated cotyledons

Rape cotyledons responded very distinctly to the kind and concentrations of the growth substances tested. Results obtained after a 2-week culture period indicate that auxins stimulated not only the increase of fresh and dry weight, but also callus formation and rhizogenesis (Figs. 1, 2). An exception were cotyledons grown on IBA, which failed to form callus and, on 2,4-D, which did not form roots. The best effects in differentiation were obtained by applying auxins at concentrations of 5 and 25 μM (Figs. 1, 2, Table 1).

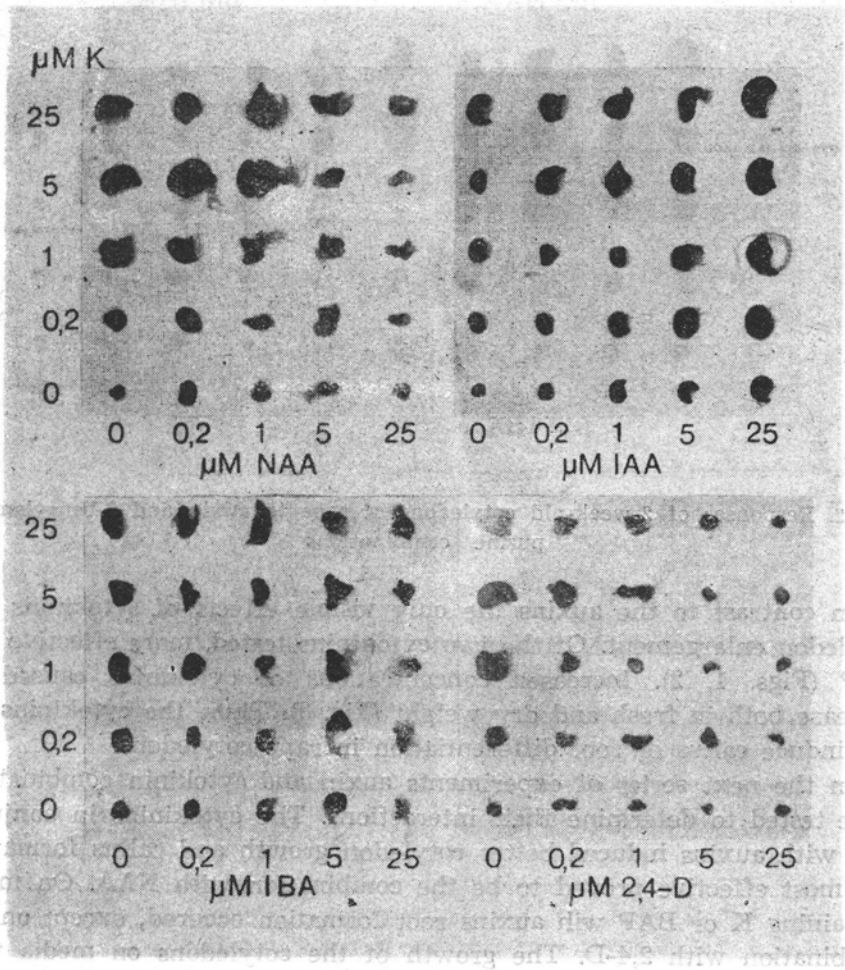

Fig. 1. Responses of 2-week-old cotyledons of rape to auxin and kinetin combinations

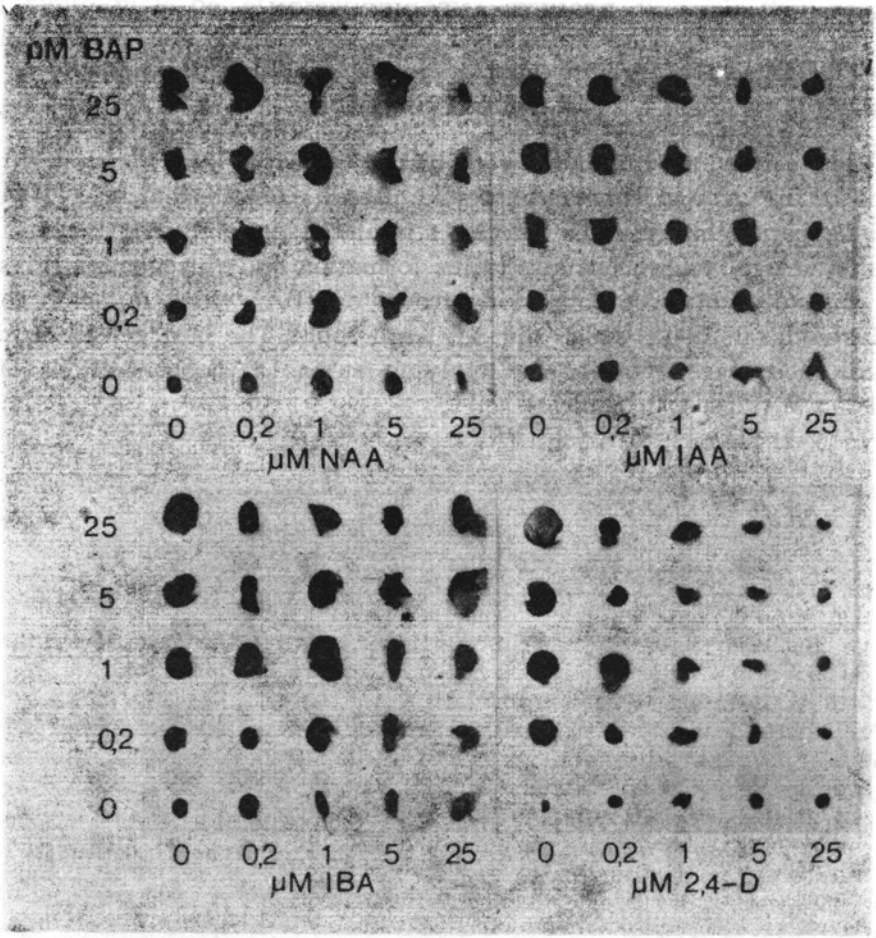

Fig. 2. Responses of 2-week-old cotyledons of rape to auxin and 6-benzylaminopurine combinations

In contrast to the auxins the only visible effects of cytokinins was cotyledon enlargement. Of the two cytokinins tested, more effective was BAP (Figs. 1, 2). Increased concentrations of cytokinins caused an increase both in fresh and dry weight (Fig. 3). Thus, the cytokinins did not induce callus or root differentiation in rape cotyledons.

In the next series of experiments auxin and cytokinin combinations were tested to determine their interactions. The cytokinins in conjunction with auxins induced better cotyledon growth and callus formation, the most effective proved to be the combination with NAA. On media containing K or BAP wih auxins root formation occured, except on the combination with 2,4-D. The growth of the cotyledons on media with 2,4-D was also suppressed and only at lower concentrations insignificant stimulatory effects were noted.

Table 1

Effect of growth substances on callus and root formation in rape cotyledons*

Cytokinins+auxins (μM)	% of cotyledons forming callus								% of cotyledons forming callus							
	K				BAP				K				BAP			
	NAA	IAA	IBA	2,4-D	NA	AIAA	IBA	2,4-D	NAA	IAA	IBA	2,4-D	NAA	IAA	IBA	2,4-D
0 + 0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0 + 0.2	0	11	0	66	0	11	0	66	50	37	0	0	50	37	0	0
0 + 1	27	22	0	100	27	22	0	100	71	88	22	0	71	88	22	0
0 + 5	100	100	0	100	100	100	0	100	100	100	92	0	100	100	92	0
0 +25	100	100	0	100	100	100	0	100	100	100	100	0	100	100	100	0
0.2+ 0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.2+ 0.2	22	0	0	80	33	0	0	100	22	0	25	0	55	0	20	0
0.2+ 1	30	0	0	90	50	0	0	100	67	29	22	0	75	0	50	0
0.2+ 5	100	0	0	100	67	0	0	100	56	50	78	0	67	22	80	0
0.2+25	100	0	0	100	100	56	0	100	100	89	100	0	100	44	67	0
1 + 0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1 + 0.2	22	0	0	80	44	22	0	100	44	0	0	0	22	11	0	0
1 + 1	44	0	0	100	50	38	0	100	67	0	10	0	44	11	0	0
1 + 5	90	20	0	100	100	44	0	100	67	56	62	0	100	44	14	0
1 +25	100	50	0	100	100	22	0	100	56	67	100	0	100	22	59	0
5 + 0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5 + 0.2	44	0	0	50	22	0	0	100	11	0	0	0	11	0	0	0
5 + 1	44	0	0	100	50	44	0	100	22	11	0	0	80	11	0	0
5 + 5	55	33	0	100	71	66	0	100	33	11	44	0	57	25	44	0
5 +25	100	0	0	100	100	12	0	100	33	78	56	0	75	25	50	0
25 + 0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
25 + 0.2	56	0	0	80	55	0	0	100	11	0	0	0	33	0	0	0
25 + 1	78	0	0	100	60	0	0	100	25	0	0	0	33	0	11	0
25 + 5	78	0	0	100	80	0	0	100	0	33	100	0	50	44	33	0
25 +25	100	0	0	100	100	0	0	100	0	22	33	0	57	30	50	0

* 12 cotyledon halves were used for each treatment

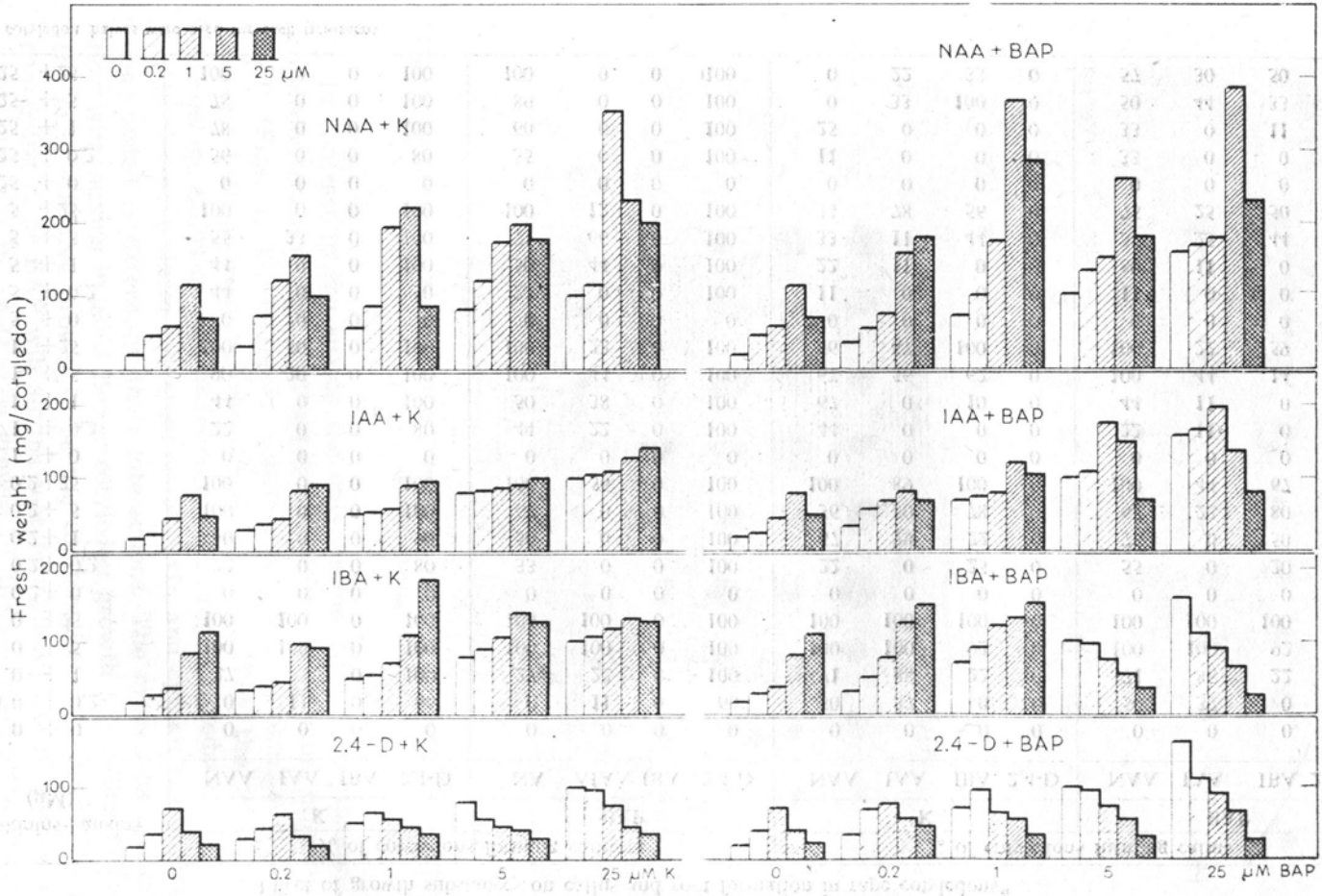

Fig. 3. Interactions of auxins and cytokinins in the growth of cotyledons (12 cotyledon halves were used for each treatment)

The cotyledons in the two-week growth period reacted with various growth and organogenetic intensity to the growth substances added to the medium. The combination of BAP with NAA was superior to the others as regards callus and root formation and the best results were obtained using 10 μM concentrations of both substances.

The two-week growth period was long enough to determine the relative morphogenetic responses of the cotyledons to auxins, cytokinins and auxin-cytokinin combinations in earlier growth stages but was insufficient to reveal the bud promoting effects. In further experiments the bud promoting effects of combinations of NAA with BAP (at 10 μM concs. each) were tested during a prolonged period of time. Buds appeared after ca. 3 weeks (Fig. 4) in ca. 50 per cent and continued at

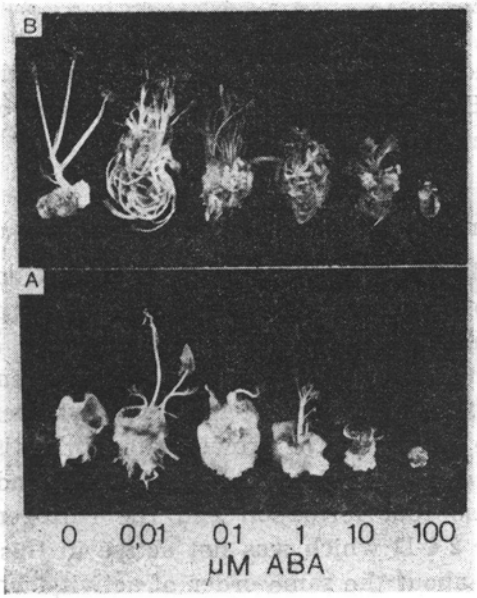

Fig. 4. Effect of ABA on differentiation of isolated cotyledons grown on medium with 10 μM NAA+10 μM BAP
A: after 3 weeks, B: after 9 weeks

this rate throughout the 9-week culture period (Table 2). In order to reveal the eventual role of ABA in the regulation of bud formation processes, it was added to medium containing 10 μM NAA + 10 μM BAP (Table 2, Fig. 3). Lower concentrations of ABA (0.01—10.0 μM) stimulated slightly bud formation in the cotyledons the highest one excepted (100 μM). The antagonistic effect of ABA regarding gibberellins, auxins and cytokinins in many bio-assays can be overcome by higher concentrations of these substances (W a r e i n g, P h i l l i p s, 1970; M i l b o r r o w, 1974). In this way might be explained the stimulatory effect

Table 2

Effect of ABA on differentiation of ioslated cotyledons on medium with 10 μM NAA+ 10 μM BAP after 9-week culture*

ABA (μM)	% of cotyledons forming plantlets
0	49
0.01	47
0.1	43
1.0	47
10.0	45
100.0	0

* 12 cotyledon halves were used for each treatment

of only lower concentrations of ABA on bud formation. A stimulatory effect of lower ABA concentrations on adventitious bud formation was also found in begonia leaves (H e i d e, 1968).

Morphogenesis of callus derived from the cotyledons

Growth and differentiation of callus derived from rape cotyledons was tested in another series of experiments. The influence of similar auxin, cytokinin and auxin-cytokinin concentrations as in the case of cotyledons was tested in order to compare the requirements of the tissue for growth and differentiation in the 4-week growth period.

In the presence of auxins, the growth rate of the tissue was insignificant and diminished in the order: NAA, IAA, 2,4-D and IBA (Fig. 5). The auxins induced also root formation and the rhizogenetic activity was similar, except for 2,4-D which was not active in this respect. The two cytokinins showed about the same order of activity which was, however, very low (Figs. 5, 6). For good growth of rape tissue, similarly as in many other systems, adequate auxin-cytokinin combinations are necessary.

When investigating the influence of auxins with cytokinins, the best growth effects proportional to the auxin concentration, were obtained on media containing NAA, and the lowest on IBA. Good growth effects were obtained also on combinations of 2,4-D with BAP. Lower concentrations of 2,4-D with cytokinins were sometimes as effective as higher ones of NAA and IAA (Figs. 5, 6, 7). All combinations of the growth substances used induced a well determined increase of tissue growth rate and, some of them, root formation. The best combination for rhizogenesis was 5 μM NAA + 5 μM BAP. The roots appeared on callus tissue in lower numbers, however, than on the cotyledons. In the course of

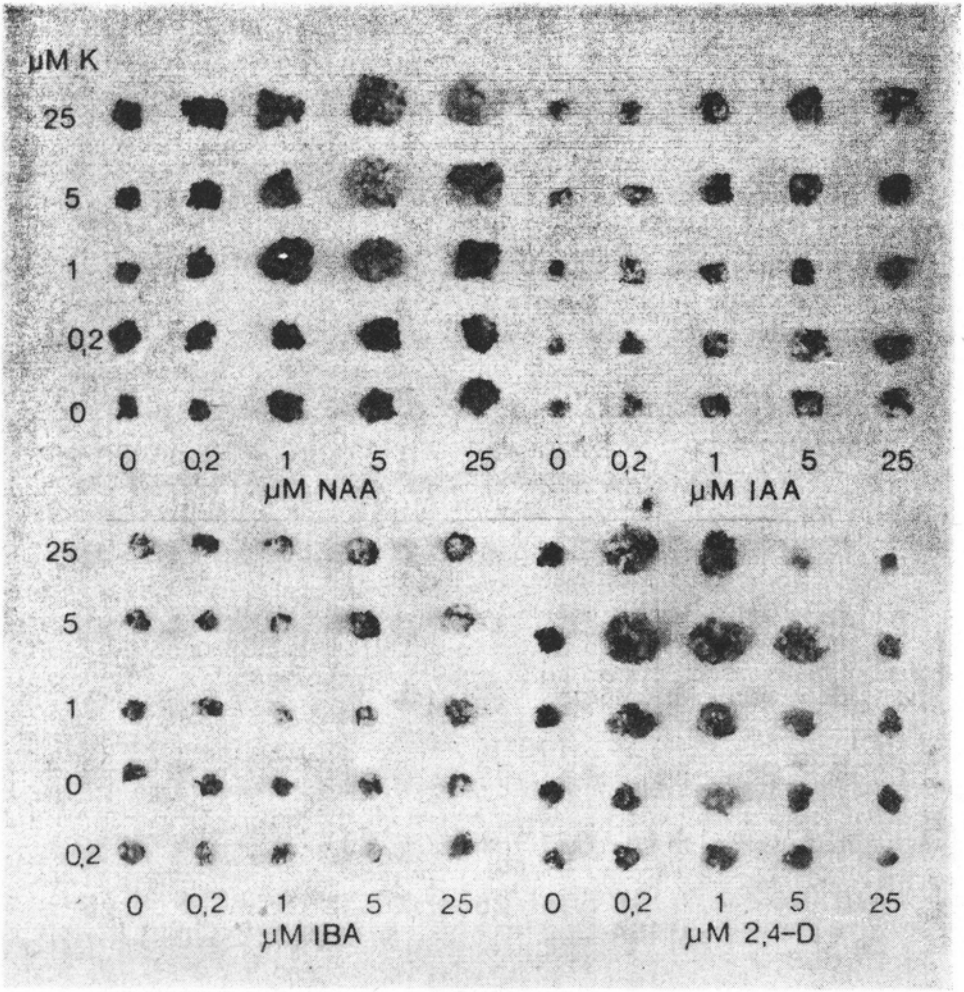

Fig. 5. Responses of 4-week-old tissue cultures of rape to auxin and kinetin combinations

subculture a decreasing ability of the tissue to form roots was observed. The tissues grown on media containing 2,4-D with cytokinins failed to form roots (Table 3). Similarly as in the case of cotyledons, the combination of auxins with kinetin was less effective on growth and rhizogenesis than that with 6-benzylaminopurine.

Thus, it was found that the callus tissue possesses a lower morphogenetic potential than the cotyledons. Whereas buds appeared on cotyledons after ca. 3-week growth, the subcultured callus tissue did not reveal this capability.

As shown, the auxin and cytokinin combinations optimal for growth of the cotyledons were also different from those optimal for the callus.

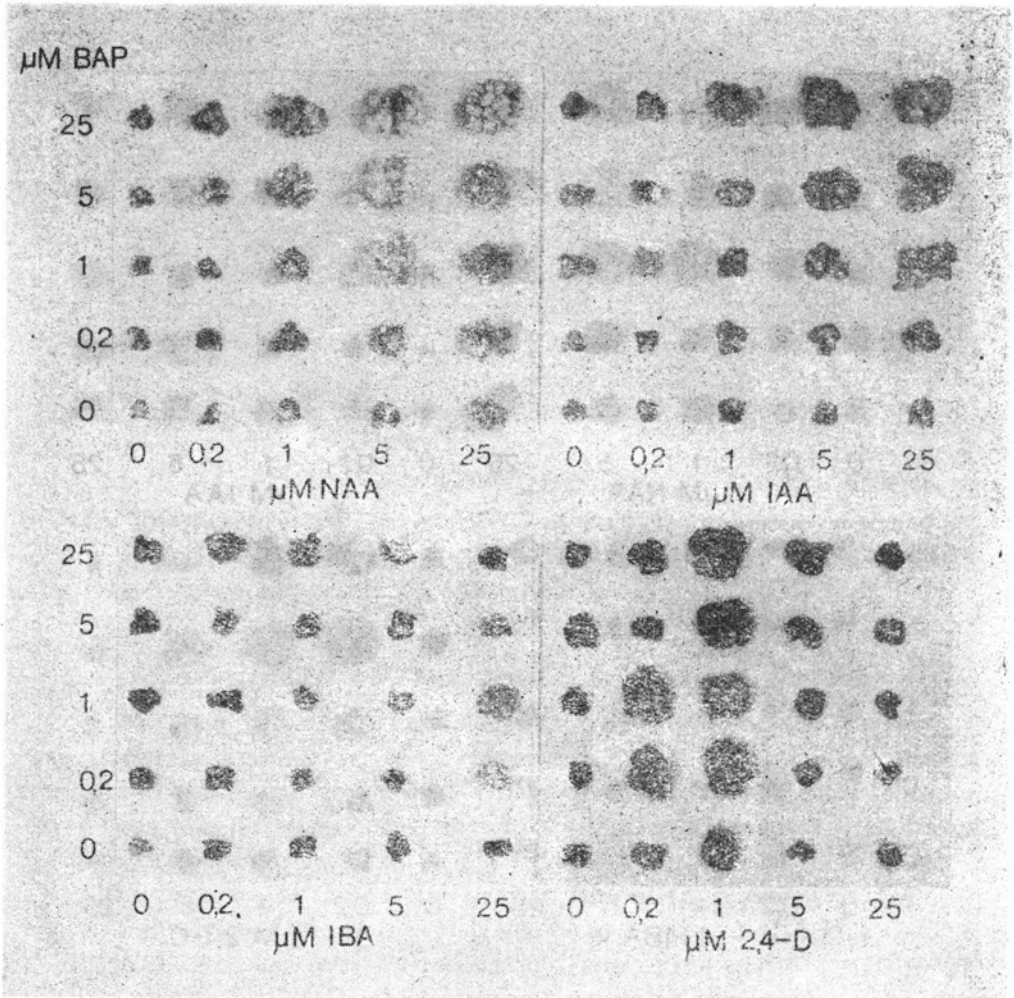

Fig. 6. Responses of 4-week-old tissue cultures of rape to auxin and 6-benzylaminopurine combinations

Relatively high increases of callus tissue were obtained on media containing 5 µM BAP with 0.2 µM 2,4-D (20-fold increases) or 25 µM NAA and on 25 µM BAP with 5 or 25 µM NAA (Fig. 3). Analogous concentrations applied to the cotyledons suppressed their growth.

Optimal medium composition for callus growth of rape was investigated by Radwan (1975). The results of his investigations suggest that by increasing the sucrose concentration and supplementing the medium with natural products such as coconut milk, casein hydrolysate or yeast extract, one can stimulate callus growth. The results of our investigations showed, however, that, to obtain tissue increments similar to those cited by Radwan (1975), the addition of such substances to

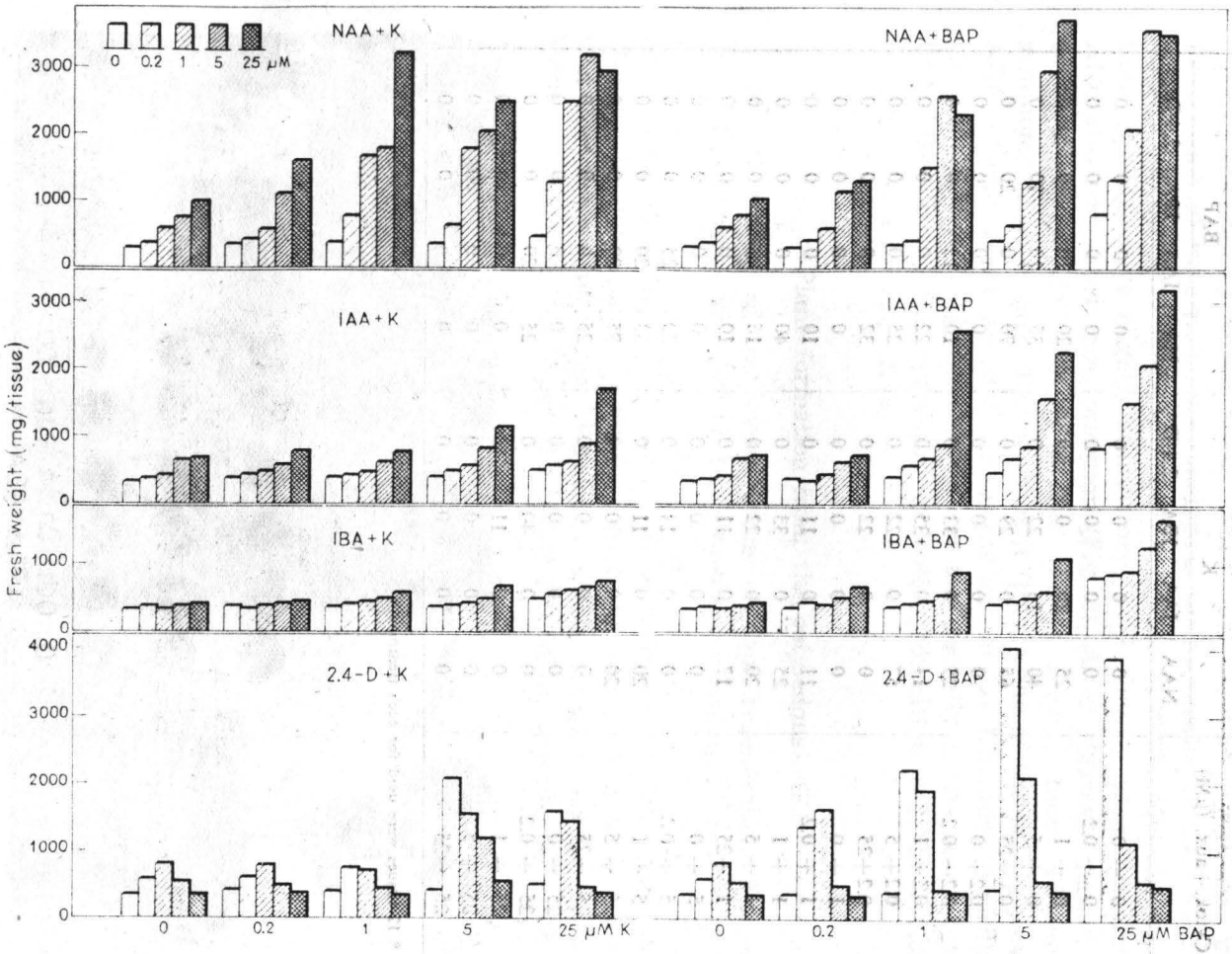

Fig. 7. Interactions of auxins and cytokinins in growth of callus tissues (12 tissues were used for each treatment)

Table 3

Effect of growth substances on root differentiation in callus tissue of rape*

Cytok. + aux. (μM)	% of callus producing roots							
	K				BAP			
	NAA	IAA	IBA	2,4-D	NAA	IAA	IBA	2,4-D
0 + 0	0	0	0	0	0	0	0	0
0 + 0.2	0	0	0	0	0	0	0	0
0 + 1	25	20	0	0	20	10	0	0
0 + 5	40	15	22	0	35	10	10	0
0 +25	65	0	29	0	70	0	20	0
0.2+ 0	0	0	0	0	0	0	0	0
0.2+ 0.2	25	0	33	0	10	12	0	0
0.2+ 1	14	0	35	0	22	0	0	0
0.2+ 5	57	0	22	0	25	0	0	0
0.2+25	0	0	22	0	32	0	0	0
1 + 0	0	0	0	0	0	0	0	0
1 + 0.2	11	0	11	0	10	0	0	0
1 + 1	25	0	33	0	40	0	0	0
1 + 5	20	0	22	0	16	0	0	0
1 +25	17	0	11	0	10	0	0	0
5 + 0	0	0	0	0	0	0	0	0
5 + 0.2	0	0	14	0	20	25	0	0
5 + 1	20	0	11	0	33	20	0	0
5 + 5	20	0	0	0	75	20	0	0
5 +25	0	0	0	0	25	0	0	0
25 + 0	0	0	0	0	0	0	0	0
25 + 0.2	0	0	40	0	25	20	0	0
25 + 1	0	0	11	0	0	0	0	0
25 + 5	0	0	0	0	0	0	0	0
25 +25	0	0	0	0	0	0	0	0

* 12 tissues were used for each treatment

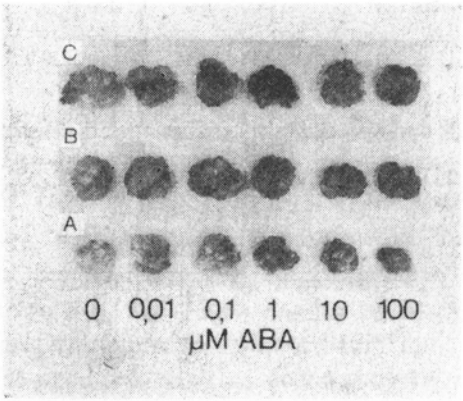

Fig. 8. Effect o ABA on the growth of callus tissue grown on medium with 10 μM NAA + 10 μM BAP

A: after 3 weeks, B: after 6 weeks, C: after 9 weeks

the medium is not necessary. It is possible to obtain still higher tissue growth rates with appropriate auxin and cytokinin combinations on strictly defined medium (e.g. 0.2 μM 2,4-D + 5 μM BAP).

The investigated influence of abscisic acid on the growth and differentiation of rape tissue was analogous to the results of Lavee and Adiri (1974) regarding apple and olive tissues. Specified low concentrations of ABA were slightly stimulatory, while higher ones inhibitory. ABA also prevented root formation (Fig. 8). After a growth period of 9 weeks on media with ABA the tissues showed more advanced symptoms of senescence than without it (this is one of the many known ABA effects). There are various suggestions in the literature regarding these interactions of growth substances and recently it has been shown that ABA affects the cytokinin nucleotide formation rate in various systems (Miernyk, 1979).

Plant formation from the cotyledons

In continuation of the investigations on rape regeneration, 9-week-old plantlets were transferred from media containing 10 μM NAA + 10 μM BAP to pots containing soil and placed in the greenhouse (Fig. 9). Plants obtained by tissue culture techniques, similary as those obtained by conventional methods, require various periods of vernalization for flower induction (Pierik, 1967; Rogozińska et al., 1979). As shown in this paper, the plants left in the greenhouse exhibit-

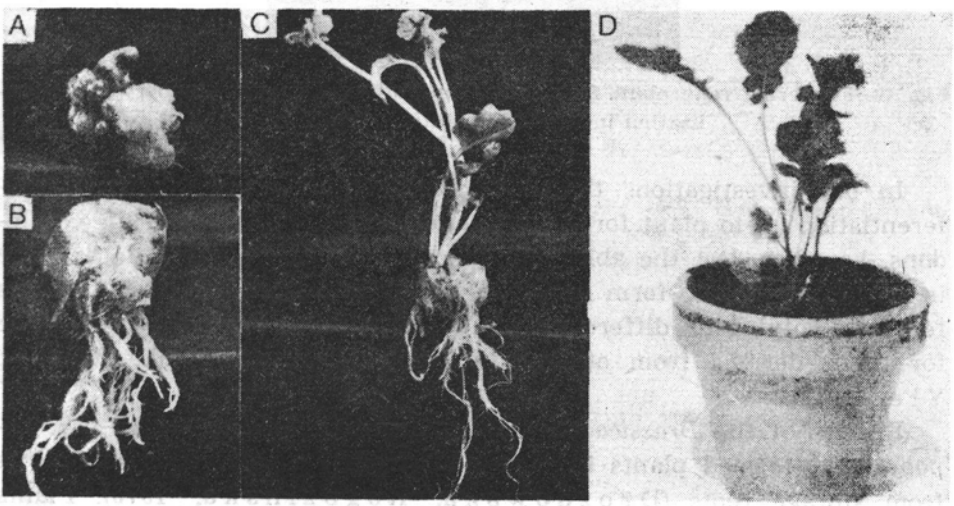

Fig. 9. Clonal multiplication of rape on medium with 10 μM NAA + 10 μM BAP

A: callus formation after 3 weeks
B: callus and root formation after 4-5 weeks
C: plantlet development after 9 weeks
D: 12-week-old plantlet

ed only vigorous vegetative growth, whereas if planted in the field where they underwent vernalization, flowered in the next spring (Fig. 10). They formed also normal seeds. Plants derived from the cotyledons were taller, with thicker stems and more inflorescences than those derived from seeds. More vigorous growth and development of plants obtained by vegetative cloning is known from the literature regarding other species.

Fig. 10. Flowering rape plant from cotyledon culture. The plant underwent vernalization in natural conditions in the field

In our investigations the cotyledons underwent organogenetic differentiation up to plant formation. Callus tissue derived from the cotyledons, however, lost the ability to shoot formation. It was able to grow in subculture and to form roots. The requirements of growth substances for rape cotyledons differentiation are somewhat different from those for tissue derived from other rape parts (K a r t h a et al., 1974a, b; Y i e, 1978).

Species of the *Brassicaceae* family, such as *Brassica napus* var. *napobrassica*, formed plants *in vitro* both from the cotyledons as well as from storage roots (D r o z d o w s k a, R o g o z i ń s k a, 1976). Plants were also obtained from cotyledons and hypocotyls of *Brassica juncea* (H u i, Z e e, 1978). The differentiation was induced by various growth substance combinations which, for each species, have to be determined. The optimal concentrations of growth substances for rape cotyledon

differentiation, as shown by the above investigations, was 10 μM NAA + 10 μM BAP. Besides, the vegetative growth stages of rape obtained by tissue culture techniques, the generative stages not tested before were also investigated.

In this paper the growth requirements of the cotyledons for complete regeneration, of plants were determined. The plants formed seeds which, sown in the soil, showed a normal growth pattern. Continuation of this work will show whether the seeds will exhibit a modified level of glucosinolates. Lowering the glucosinolate content by applying the tissue culture method can be expected to bring a new potential in rape breeding.

REFERENCES

Afzalpurkar A. B., 1974. Fette, Seifen, Anstrichmittel 76: 550-552.

Drożdowska L., Rogozińska J. H., 1975. Hod. Roślin. Aklim. Nas. 19. 29-34.

Drozdowska L., Rogozińska J. H., 1976. Acta Univ. Nicolai Copernici, Biologia 18: 105-111.

Heide O., 1968. Nature 219: 960-961.

Hui L. H., Zee S. Y., 1978. Z. Pflanzenphysiol. 89: 77-80.

Kartha K. K., Michayluk M. R., Kao K. N., Gamborg O. L., Constabel F., 1974a. Plant Sci. Lett. 3: 265-271.

Kartha K. K., Gamborg O. L., Constabel F., 1974b. Physiol. Plant. 31: 217-220.

Lavee S., Adiri N., 1974. In: „Plant Growth Subst. 1973": 1141-1148. Tokyo, Hirokawa.

Miernyk J. A., 1979. Physiol. Plant. 45: 63-66.

Milborrow B. V., 1974 Ann. Rev. Plant Physiol. 25: 260-307.

Murashige T., Skoog F., 1962. Physiol. Plant. 15: 473-497.

Pierik R. L. M., 1967. Ph. D. Thesis, Agric. Univ. Wageningen, H. Veenman and Zonen 67.

Radwan S. S., 1975. Fette, Seifen, Anstrichmittel 77: 19-20.

Rogozińska J. H., Drozdowska L., in press.

Rogozińska J., Kotowska U., Gośka M., 1979. Hod. Roślin Aklim. Nas. 23: 1-7.

Wareing P. F., Phillips I. D. J., 1970. The Control of Growth and Differentiation in Plants. Pergamon Press Ltd.

Yie S. T., 1978. In Vitro 14: 356.

Organogeneza i tworzenie roślin w kulturach liścieni i kalusa rzepaku

Streszczenie

Liścienie rzepaku hodowano przez 2 tygodnie na pożywce Murashige i Skoog'a z auksynami, cytokininami i ich kombinacjami oraz z kwasem abscysynowym. Tworzenie kalusa zachodziło na pożywce z 2,4-D, NAA i IAA oraz na ich kombinacjach z K lub BAP. Liścienie tworzyły korzenie na pożywce z NAA, IAA i IBA i na kombinacjach tych auksyn z cytokininami. Obecność 2,4-D w po-

żywce chociaż stymulowała tworzenie zbitego kalusa, wpływała hamująco na tworzenie korzeni.

Kalus pochodzący z liścieni miał nieco inne wymagania wzrostowe, a zdolność do tworzenia korzeni zmniejszała się w trakcie jego hodowli. Niższe stężenia ABA stymulowały wzrost kalusa, podczas gdy wyższe hamowały, podobnie jak w przypadku liścieni.

Z liścieni hodowanych na pożywce z NAA + BAP, po 3 tygodniach wzrostu różnicowały się pączki. Rozwinięte z nich roślinki przesadzano do gleby. Rośliny poddane jaryzacji tworzyły kwiatostany i nasiona.