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In Vitro Development from Leaf Explants of Sugar Beet (*Beta vulgaris* L). Rhizogenesis and the Effect of Sequential Exposure to Auxin and Cytokinin

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Adventitious root development in lamina and midrib-petiole junction explants of sugar beet cv. Primo was investigated using scanning electron microscopy and light microscopy. Primordia developed close to the vascular strands and areas of newly dividing cells (meristematic centres) were seen adjacent to the intrafascicular cambium after 2 d incubation on medium containing 30 mg l^{-1} 1-naphthalene acetic acid. Clearly defined primordia were visible at 4 d and the first roots had emerged by 6 d. A minimum of 24 h exposure to NAA was necessary for root induction. Four days on NAA caused twice as many roots to be initiated but more prolonged exposure (5 and 10 d) inhibited root development. Root initiation continued after transfer to medium containing no plant growth regulators, new primordia appearing as the older ones extended as roots. Attempts were made to modify the development of primordia by sequential culture on cytokinin after induction by auxin. Incubation on N6-benzylaminopurine within 48 h of exposure to NAA disrupted the development of primordia and roots but did not induce shoot formation.

Key words: *Beta vulgaris*, sugar beet, *in vitro* culture, leaf explants, rhizogenesis, morphogenetic plasticity.

INTRODUCTION

Plant tissue culture is widely used in plant propagation and is an essential tool in the application of molecular genetics to crop improvement. Callus development is readily obtained from many species but the regeneration of new plants, either from callus or directly from the original explant, is more problematic. Regeneration may be achieved through organogenesis or somatic embryogenesis and progress has been made in defining the conditions required for, and the physiological and biochemical changes accompanying these types of development, (Ammarito, 1987; Christianson, 1987; Thorpe, 1990). However, not all species can be propagated in this way and many crop plants are reluctant to initiate shoots in culture.

Our understanding of the processes involved in organogenesis is still incomplete. We do not know how meristems are induced *de novo* nor the factors which regulate their development into roots or shoots. Is the fate of a meristem determined at its inception or may the early stages of organogenesis be plastic, meristems becoming committed to develop into roots or shoots by virtue of the conditions prevailing at some critical stage?

Bonnett and Torrey (1965) found that young bud and root primordia in cultured *Convolvulus arvensis* roots were histologically indistinguishable and that buds could be induced to develop at presumptive root sites. They proposed that the increased number of buds observed when cultures were transferred to medium lacking auxin after several days in 10^{-5} M IAA 'may be due to the initiation of a large number of primordia by auxin, which can then develop into either buds or roots depending on subsequent auxin levels'. Others have described the transformation of cultured root tips into shoots in several genera including *Neottia nidusavis*, *Anthurium longifolium*, *Selaginella* and *Vanilla planifolia*, (Champagnat, 1971; Peterson, 1975; Wochok and Sussex, 1975; Philip and Nainar, 1986, 1988; Philip and Padikkala, 1989). However, Sharma, Bhojwami and Thorpe (1990) found that shoots and roots which appeared at the same morphological position in cotyledons of *Brassica juncea* developed from different tissues.

Christianson and Warnick (1983, 1984, 1985) suggested that organogenesis proceeds through three sequential stages: (1) the acquisition of competence to respond to a particular inductive signal, (2) induction, and (3) morphogenic differentiation and development. Working with *Convolvulus arvensis* leaf explants, they found that competence was achieved on a range of culture media. Induction of roots occurred on medium supplemented with indole-3-butyric acid (IBA) and a combination of 2-isopentenyladenine (2-iP) and indole-3-acetic acid (IAA) resulted in shoot induction. The subsequent development of morphologically distinct roots and shoots took place on media containing any or no plant growth regulators.

Sugar beet is a recalcitrant species and previous attempts to regenerate plants from normal leaf tissue have met with only limited success (Saunders and Doley, 1986; Detrez, Sangwan and Sangwan-Norreel, 1989). Material taken from aseptically cultured shoot apices was more responsive, the development of shoots as well as roots being determined by the nature, concentration, ratio and/or sequence of plant growth regulators provided (Tetu, Sangwan and Sangwan-Norreel, 1987; Detrez *et al.* 1988; Konwar and Coutts,

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1990). Gürel (1991) obtained roots but no shoots on lamina explants from pot-grown plants of cv. Primo when cultured on either MS medium or the revised medium of Freytag *et al.* (1988) supplemented by auxin and/or cytokinin.

In the current work, our aim was to regenerate complete plants from normal leaf tissue. We first induced meristematic activity in the explants by a short exposure to auxin and then attempted to modify the subsequent development of primordia by treatment with cytokinin, either directly or after a period on medium containing no plant growth regulators. This paper begins by describing the normal course of rhizogenesis as observed by a combination of scanning electron microscopy (SEM) and light microscopy, and then examines the induction and initiation of meristems and their subsequent development on media supplemented by l-naphthaleneacetic acid (NAA) and N6-benzylamino purine (BAP).

MATERIALS AND METHODS

Material and culture conditions

Sugar beet plants cv. Primo were grown in a peat-based compost in a controlled environment room, (25 °C and 16 h photoperiod under warm white fluorescent tubes supplying 220–240 μE m^{-2} s^{-1}). The youngest fully expanded leaf was removed from 20–30 d old plants and surface sterilised by submergence in 5% Domestos (a commercial bleach containing 9·5% sodium hypochlorite) for 20 min, followed by several rinses with sterile distilled water. Explants were prepared either by taking 8 mm discs from the middle segment of the lamina (avoiding the distal and proximal ends) using a sharp cork borer, or by cutting 5 mm pieces from the midrib–petiole junction region with a scalpel. As the leaf lamina extends for a short distance along the petiole, the point of insertion of the lowest primary vein was used as a marker and not more than four junction explants were taken on either side of this position. Explants were placed with the abaxial surface in contact with MS medium (Murashige and Skoog, 1962) containing 3% sucrose and 0·8% Oxoid No. 3 agar, pH 5·9, which had been sterilized by autoclaving for 5 min at 103·5 kPa. After sealing the plastic petri dishes with Parafilm, the cultures were incubated at 25±2 °C in low light conditions (40–50 μE m^{-2} s^{-1} and 16 h photoperiod).

Preparation for SEM examination

Lamina discs were fixed in 1% osmium tetroxide overnight. They were then washed twice with distilled water, dehydrated in 20, 40, 60 and 80% acetone and left in 100% acetone overnight. Samples were dried in CO_2 using a critical point drying apparatus and sputter coated with gold prior to examination with a CamScan scanning electron microscope.

Clearing and staining

The method of Hackett and Stewart (1969) was used for the detection of meristematic activity. Material fixed in formalin-acetic acid-alcohol (FAA) was transferred to 70% lactic acid and incubated at 60 °C overnight or until transparent. After rinsing three times with distilled water, the samples were stained in aceto-carmine for 30 min. They were then rinsed well in distilled water and dehydrated in 50, 70, 95 and 100% alcohol before transfer to 50/50 ethyl alcohol/methyl benzoate. Two further changes of 100% methylbenzoate were used before observation in the same solvent under a low power stereo microscope, (×3–30).

Embedding and sectioning for light microscopy

Samples fixed in FAA were rinsed twice in distilled water, dehydrated in 50, 75, 90 and 100% ethyl alcohol and embedded in JB4 resin (PolySciences, UK). Sections were cut using a glass knife and a Sorvall 'Porter-Blum' ultramicrotome, (Smith and Wren, 1983). After staining with 0·05% Toluidine Blue in phosphate buffer pH 6·6, the sections were mounted in tap water.

RESULTS

Establishment of optimal culture conditions

As material from pot-grown cv. Primo had not previously been cultured, it was necessary to determine the optimal conditions for meristem development. Continuous incubation of lamina discs on media containing NAA showed that the greatest number of roots was obtained on media containing 10 and 30 mg l^{-1} NAA, (7·8 and 7·2 roots per explant respectively after 19 d incubation compared with 0·2 roots per explant in the absence of NAA). BAP at concentrations of 0·1–1·0 mg l^{-1} inhibited root development but stimulated callus formation. Explants taken from the midrib–petiole junction region rooted more readily than lamina discs (Table 1) and when cut paradermally into three slices, the middle slice formed roots more rapidly and over a longer period than the upper or lower piece (Table 2). The

TABLE 1. *Comparison of root formation by lamina discs and midrib-petiole junction segments incubated on MS medium containing 1·0 mg l^{-1} NAA for 30 d (75 replicates per treatment)*

	Days to first root formation	% explants with roots	Mean number of roots per explant
Lamina discs	16	33	1·2±0·28
Midrib-petiole junction segments	9	75	2·9±0·44

TABLE 2. *Comparison of root formation by upper, middle and lower slices of midrib–petiole junction segments after 5 d on MS medium containing 30·0 mg l^{-1} NAA followed by incubation on basal medium (60 replicates per treatment)*

	Mean number of roots per explant at		
	8 d	13 d	17 d
Upper	0·1±0·03	1·1±0·19	1·4±0·52
Middle	1·6±0·31	3·5±0·69	4·6±1·19
Lower	0·1±0·04	0·5±0·14	0·5±0·14

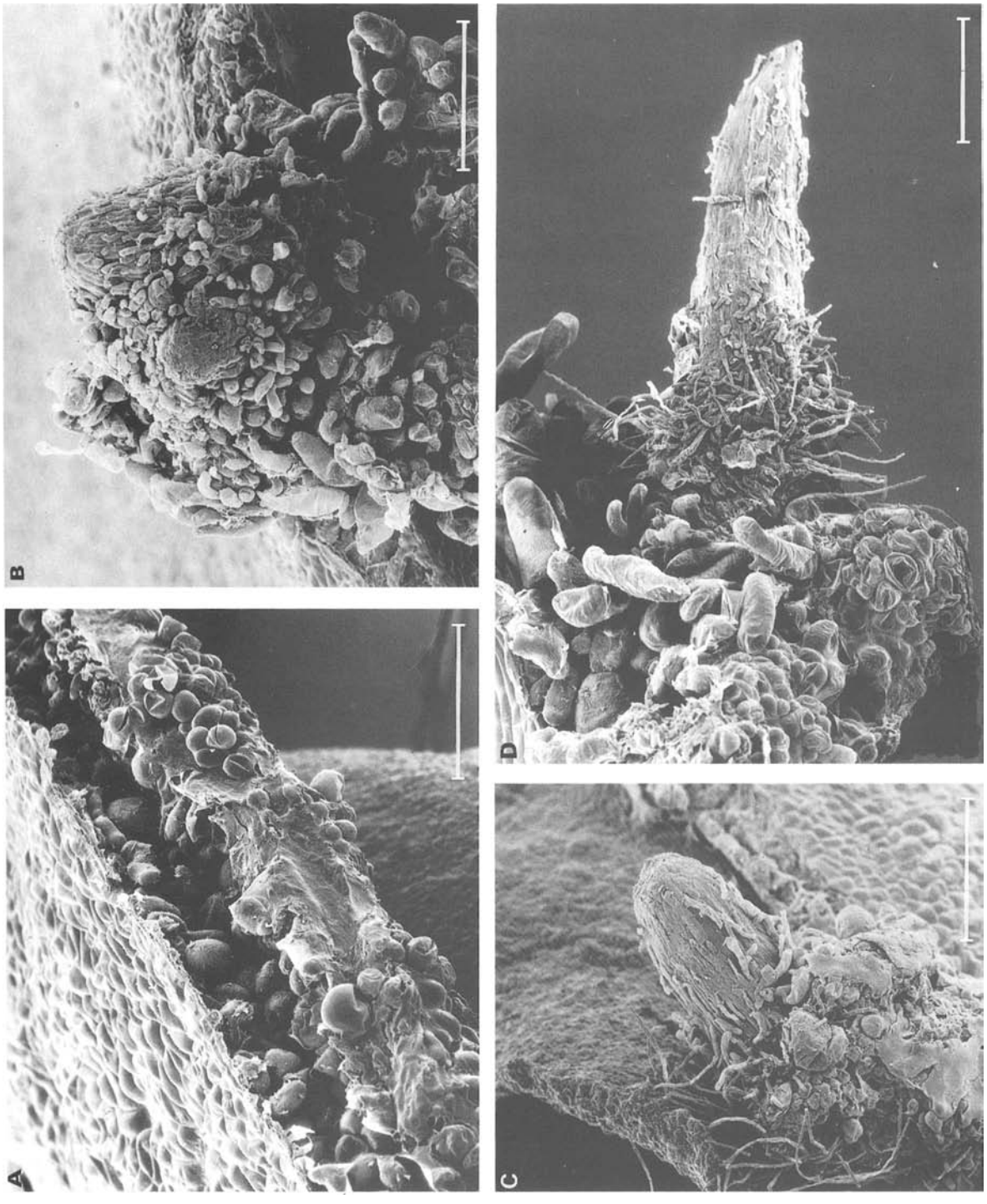

FIG. 1. A, The edge of a freshly cut (0 d) lamina disc, showing intact mesophyll cells between the upper and lower epidermis. B, cut edge of a lamina disc cultured on 1·0 mg l^{-1} NAA for 14 d. A large root tip is emerging from a mound of tissue with another smaller root tip on the left. C, A well-developed root tip after 15 d culture. D, A longer root with root hairs after 16 d and showing giant cells protruding from the explant. Bars = 300 μm.

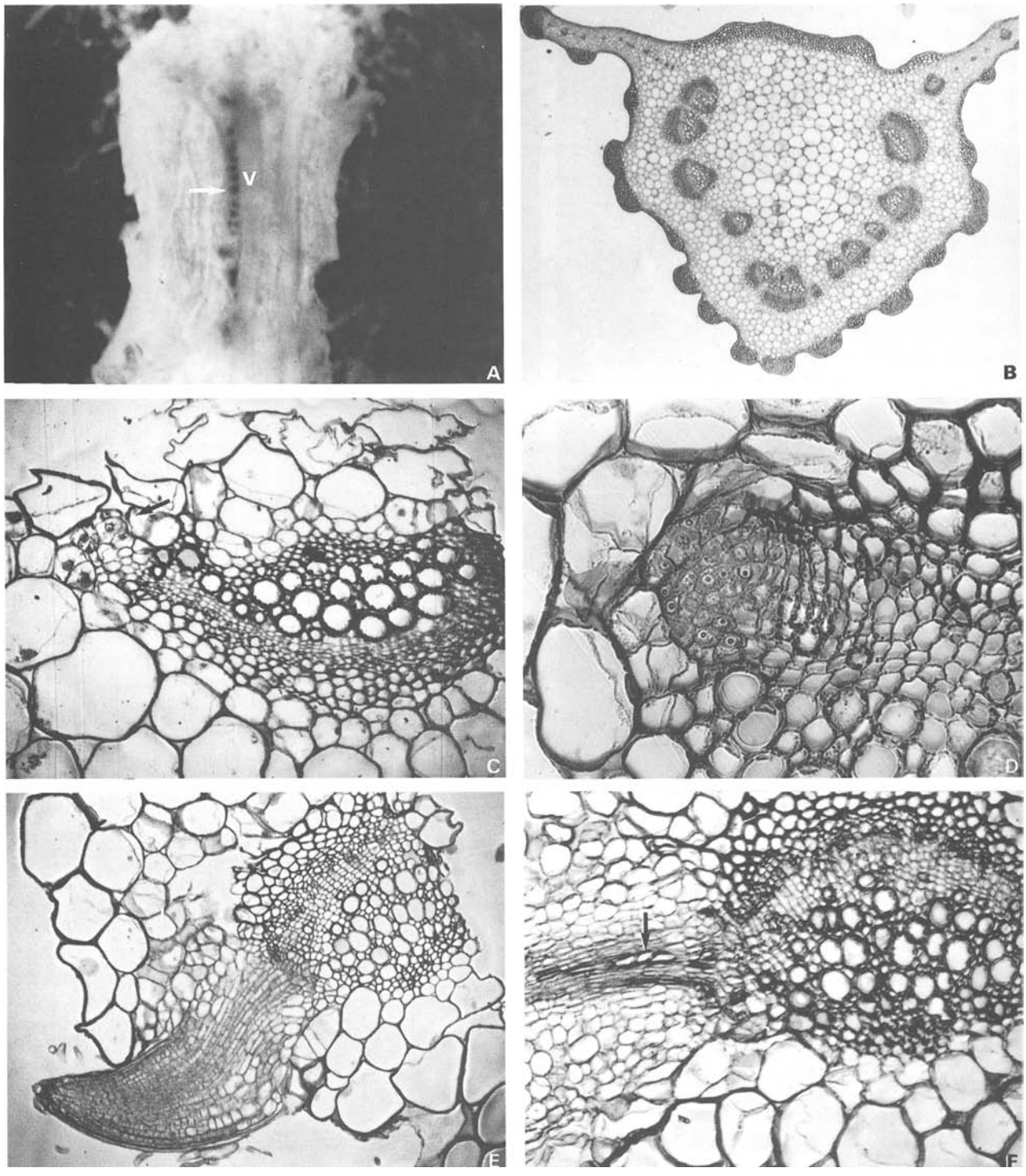

FIG. 2. A, Meristematic activity in a cleared midrib–petiole junction segment after 5 d on 30 mg l^{-1} NAA followed by BM for 10 d. Patches of aceto-carmine stained cells are visible (arrow) adjacent to a large vascular bundle (V). × 10. B, Transverse section through a midrib–petiole junction segment. × 15. C, Transverse section through a middle slice of a midrib-petiole junction segment after 48 h on 30 mg l^{-1} NAA. Note the appearance of new cells with prominent nuclei forming a meristematic centre (arrowed) adjacent to the cambial zone of a large vascular bundle. × 25. D, Transverse section through a middle slice showing a developing primordium after 4 d on 30 mg l^{-1} NAA. × 100. E, Transverse section through a middle slice showing a well-developed root after 8 d in culture (4 d on 30 mg l^{-1} NAA followed by 4 d on BM). × 25. F, Longitudinal section through the base of an adventitious root showing the differentiation of vascular tissue (arrowed) and connection to the vascular bundle of the explant after 4 d on 30 mg l^{-1} NAA followed by 6 d on BM. × 40.

addition of 2 mg l^{-1} silver nitrate to the culture medium increased the number of roots formed by middle segments by 148%.

Observation of root development by SEM

Lamina discs cut from interveinal tissue were cultured on MS medium containing 1·0 mg l^{-1} NAA and examined at intervals. Intact mesophyll cells could be seen between the upper and lower epidermis of freshly cut material (0 d), although there had been some compression of the tissues (Fig. 1A). There was no visible change in appearance after 24 h but a few new cells were seen at the cut edge of some discs by 48 h. Cell division continued and a small amount of callus was present after 7–9 d. By 13–14 d, one or more root tips could be seen emerging from mounds of tissue close to the cut edge of the disc (Fig. 1B). These grew rapidly, reaching 5–10 cm in length and with many lateral roots after 6–12 d. A conspicuous tuft of hairs preceded the appearance of each new root and long root hairs developed on the older roots (Fig. 1D). Hyphal-like cells on the surface of the roots may have been derived from the root cap (Fig. 1C and D). Many of the mesophyll cells in the leaf disc also enlarged and giant cells could be seen around the base of the elongating roots (Fig. 1B and D).

Location of meristematic activity in cleared segments

Midrib–petiole junction segments were incubated on medium containing 30 mg l^{-1} NAA for 5 d and then transferred to basal medium (BM) for 10 d. Segments were divided longitudinally into two pieces before fixing, clearing and staining with aceto-carmine to locate meristematic activity.

Areas of heavily stained cells were visible adjacent to the opaque vascular strands in the tissue (Fig. 2A). Many of these failed to develop further in intact segments and only those near the cut ends normally emerged as roots. Occasionally, however, the segments split open and when this happened, more roots grew out from the exposed tissue. Removing tissue from the adaxial and abaxial sides before culturing the middle slice also caused roots to develop along the entire length of the segment.

Light microscopy of root development

Middle slices were cultured on MS medium containing 30 mg l^{-1} NAA for 4 d before transfer to BM and samples were fixed at 2 d intervals. In transverse section, the midrib–petiole junction region is triangular in shape and contains several large and some smaller vascular bundles, with very small bundles in the laminar flanges (Fig. 2B). The collateral bundles are surrounded by parenchyma tissue and there is a continuous layer of collenchyma beneath the epidermis on the adaxial side and in the projecting ridges on the abaxial surface.

After 48 h incubation on 30 mg l^{-1} NAA, small groups of new cells with prominent nuclei and nucleoli were seen close to some of the larger vascular bundles and adjacent to the cambium (Fig. 2C). By 4 d, more divisions had occurred and young primordia were present (Fig. 2D). Root tips were seen growing through the cortex and had emerged from the cut surface by 8 d (Fig. 2E) and differentiation of the stele and connection to the vascular bundle of the explant had occurred after 10 d (Fig. 2F). Meristematic activity also occurred occasionally in the parenchyma at some distance from vascular tissue.

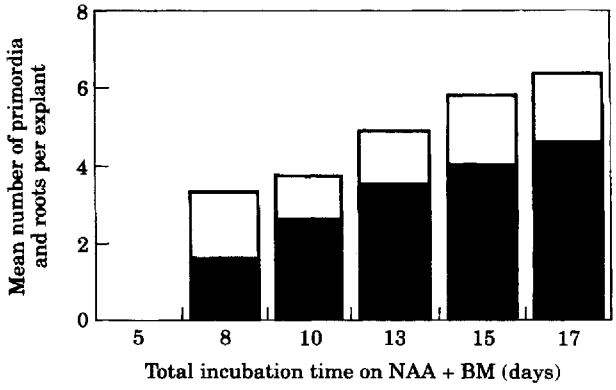

FIG. 3. Mean numbers of primordia (□) and roots (■) developing from the middle slice of midrib–petiole junction explants cultured on 30 mg l^{-1} NAA for 5 d followed by incubation on BM (20 explants per treatment).

Development in sequential culture regimes

Development on basal medium following exposure to auxin. Middle slices of midrib-petiole junction segments were incubated on medium containing 30 mg l^{-1} NAA for 5 d and then transferred to hormone-free medium (BM). The numbers of primordia and emerged roots were determined by clearing the tissue at intervals over 17 d. (In Figs 3, 4 and 7, the term primordia refers to the total number of meristematic centres and older primordia still within the explant tissue).

No primordia were detected during the 5 d on NAA but primordia and roots were present by 8 d. The number of emerged roots increased with length of incubation whilst the number of primordia remained constant at just under two per explant (Fig. 3), indicating that meristematic centres continued to be initiated throughout the culture period.

Length of exposure to auxin and time of induction. Shorter incubation times on NAA were tested by culturing middle slices on 30 mg l^{-1} NAA for periods of 0·5 to 5·0 d prior to transfer to BM.

Observation of cleared tissues showed that a minimum of 24 h exposure to NAA was needed for induction to occur and means of 0·9 primordia and 1·5 roots per explant were obtained after a further 7 days in basal medium (Fig. 4). More primordia (3·4) were produced after 4 d exposure to NAA but extending this to 5 d was no more effective than 1–3 d of auxin treatment. No root initiation had taken place during the 5 d incubation on NAA, the first primordium being detected after one further day on basal medium and the first root appeared 2 d later (8 d in total).

Effects of auxin–cytokinin transfer regimes. Attempts were made to divert primordial development from roots to

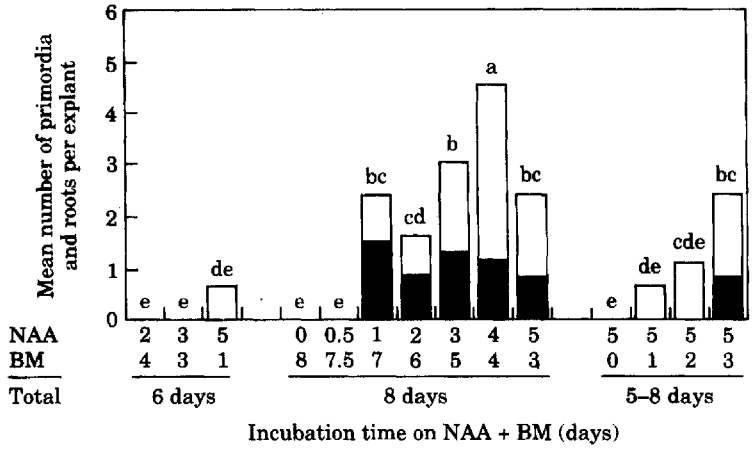

FIG. 4. Mean numbers of primordia (□) and roots (■) in the middle slice of midrib–petiole junction explants cultured on 30 mg l^{-1} NAA for 1–5 d followed by BM for 1–8 d. Values with the same letter (primordia plus roots) are not significantly different at $P = 0.05$ (20 explants per treatment).

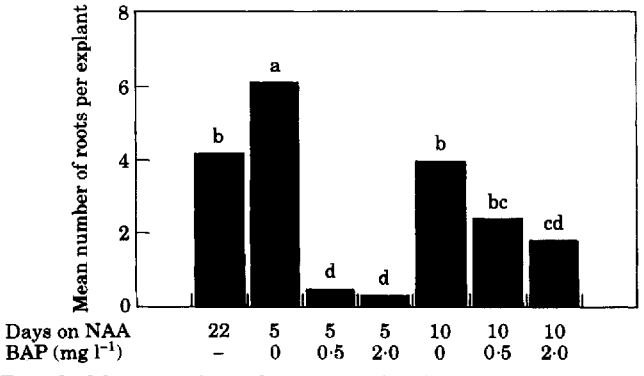

FIG. 5. Mean number of roots produced by middle slices of midrib–petiole junction explants cultured on medium containing 30 mg l^{-1} NAA for 5 or 10 d prior to transfer to media containing 0, 0·5 and 2·0 mg l^{-1} BAP. Results obtained after 22 d culture (5 + 17 d or 10 + 12 d on NAA + BAP). Values with the same letter are not significantly different at $P = 0.05$ (25 explants per treatment).

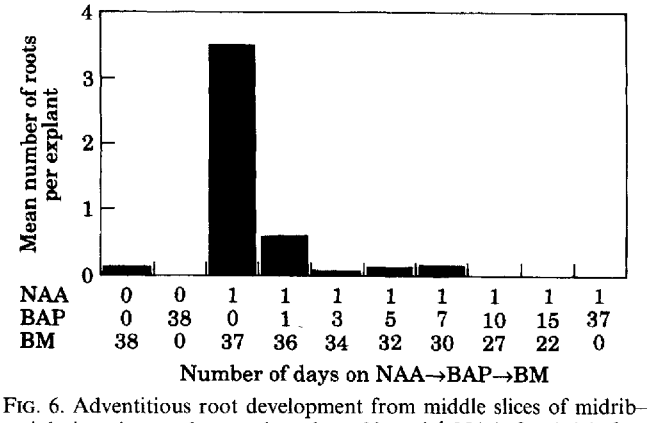

FIG. 6. Adventitious root development from middle slices of midrib–petiole junction explants cultured on 30 mg l^{-1} NAA for 1 d before transfer to 5 mg l^{-1} BAP for 1, 3, 5, 7, 10, 15 or 37 d and then to BM (38 d incubation, 20 explants per treatment).

shoots by transferring middle slices to medium containing cytokinin after an initial incubation with auxin. In the first experiment, explants were transferred to media containing 0·5 and 2·0 mg l^{-1} BAP after 5 or 10 d on 30 mg l^{-1} NAA.

The results shown in Fig. 5 demonstrated that prolonged exposure to auxin inhibited root development. Explants cultured on medium containing 30 mg l^{-1} NAA for 10 d produced 33% fewer roots than those which had been transferred to basal medium after only 5 d on NAA. Exposure to BAP after 5 d on NAA almost completely suppressed root formation. The cytokinin treatment was less inhibitory after 10 d on auxin but mean root number was still reduced by 40 and 60% respectively when these explants were transferred to 0·5 and 2·0 mg l^{-1} BAP.

In a subsequent experiment, explants were cultured on medium containing 30·0 mg l^{-1} NAA for only 1 d and were then transferred to medium containing 5·0 mg l^{-1} BAP for varying lengths of time prior to the final incubation on basal medium. Control explants were maintained on either basal medium or BAP throughout the 38 d culture.

BAP again suppressed the development of roots after induction on NAA. Even a single day on BAP reduced mean root number from 3·5 to less than 1·0 and explants cultured on BAP for three or more days produced scarcely any roots, (Fig. 6).

In a third experiment, a period on basal medium was intercalated between the auxin and the cytokinin treatment and 2·0 mg l^{-1} $AgNO_3$ was included in each of the culture media. Explants were incubated on basal medium for 1–4 d after a period of 4 d on 30·0 mg l^{-1} NAA. They were then exposed to 1·0 mg l^{-1} BAP for 1 d and transferred to basal medium until harvested at 15 d.

Explants which had not been exposed to BAP produced large numbers of roots (4·0) and primordia (23·0), demonstrating the stimulatory effect of $AgNO_3$, (Fig. 7). Transfer to medium containing BAP immediately after the auxin treatment reduced the number of roots and primordia to 1·3 and 5·0, respectively. One day on basal medium before transfer to BAP had no effect but extending this reduced the susceptibility to cytokinin and allowed more primordia and roots to develop.

DISCUSSION

Root development was studied in order to understand the process of organogenesis in sugar beet leaf explants. Scanning electron microscopy of lamina explants showed

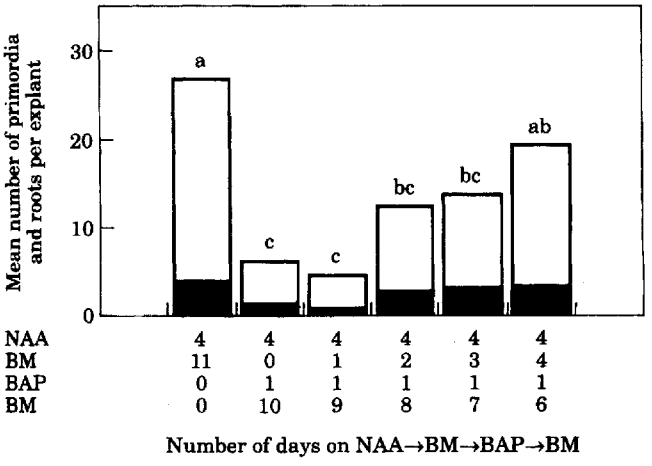

FIG. 7. Primordium (□) and root (■) development from the middle slice of midrib–petiole junction explants after 15 d culture. All explants were cultured on 30 mg l^{-1} NAA for 4 d followed by incubation on BM for 1–4 d before 1 d on 1·0 mg l^{-1} BAP and then back to BM. All media contained 2 mg l^{-1} $AgNO_3$. Values with the same letter (primordia plus roots are not significantly different at $P = 0{\cdot}05$) (20 explants per treatment).

root tips emerging from the cut margin surrounded by long hairs and light microscopy located primordia close to the vascular bundles of midrib–petiole segments. Localized cell divisions resulted in the appearance of meristematic centres within 2 d but it was impossible to determine whether the first divisions occurred in the cambium or in the parenchyma adjacent to the cambium. White and Lovell (1984) had similar difficulty with hypocotyl cuttings of *Griselinia littoralis* and *G. lucida*. Vascular parenchyma and cortical cells near the vascular tissue were implicated in petiole segments of *Brassica juncea* and *Pereskia grandifolia* (Sharma and Bhojwami, 1990; Carvalho, Monteiro and Dietrich, 1989), and in *Phaseolus vulgaris*, cell divisions also occurred in the region between the xylem and phloem (Gramberg, 1971). Well differentiated primordia were present in sugar beet explants after 4 d in culture. These grew through the explant tissue to emerge as root tips by 6 d and by 8 d the roots had elongated and formed root hairs. Vascular tissues had differentiated within the roots and connections to vascular bundles in the explant were visible by 10 d. The absence of callus near the developing primordia indicated that root development was an example of direct organogenesis.

Some reports suggest that very young primordia are plastic and may be induced to follow divergent developmental routes under particular cultural regimes. We hoped that it would be possible to induce meristematic activity in midrib-petiole junction explants and then to modify development so that shoots were formed instead of, or in addition to roots.

The length of exposure to auxin was critical for the induction of meristematic activity, a minimum of 24 h on 30 mg l^{-1} NAA being necessary for primordia to develop during the subsequent incubation on basal medium. Initiation continued over several days, more meristematic centres appearing as the older primordia developed into roots. This was considered to be an advantage, as it was not known at what stage or for how long the primordia might remain plastic.

Cytokinin inhibited root formation, its effectiveness depending on the length of exposure to auxin and the duration of any intervening period on basal medium. BAP given immediately or 1 d after 4 d induction on NAA reduced both primordial initiation and root development. Two days on basal medium between the auxin and cytokinin treatments resulted in fewer primordia but nearly as many roots as with auxin alone, and BAP had no effect when supplied after four intercalated days. This suggests that the early stages of primordium initiation were more susceptible to cytokinin than subsequent root development. Confirmation was obtained by microscopic examination of explants from two treatments: 4 d on NAA followed by 11 d on basal medium was compared with 4 d on NAA followed by 1 d on BAP and then 10 d on basal medium. Explants transferred directly from NAA into basal medium showed the organised development described earlier, some roots having already emerged and other root tips growing through the explant, with younger primordia and meristematic centres visible near the vascular tissue. In contrast, explants given a single day on 1·0 mg l^{-1} BAP before transfer to basal medium showed areas of dividing cells beneath the cut surface and within the parenchyma but none of the organised meristematic activity which preceded root development.

These experiments have shown that exposure to cytokinin effectively blocks root development in sugar beet tissue but without inducing shoot formation. Diversion of development should have been possible if the early stages of primordium development were plastic, i.e. competence having been obtained without determination *sensu* Christianson and Warnick. In some materials however, competence and determination may be achieved under the same culture regime and it is then difficult to distinguish between the two states. This may have been the situation in our experiments, where root induction was complete at some time between 12 and 24 h incubation on 30 mg l^{-1} NAA. Alternatively, it is possible that organogenesis in sugar beet leaf explants may not proceed via the competence–determination–initiation sequence but that development may be programmed towards roots or shoots from the beginning. Tran Than Van (1981) found no evidence for the transformation of root primordia into shoots in tobacco thin cell layer cultures, where conditions which favoured the development of shoots appeared to suppress root development and *vice versa*. Attfield and Evans (1991*a*, *b*) also reported differences in the origin of adventitious roots and shoots in lamina explants of tobacco, root formation occurring directly from leaf tissue within 24 h whilst shoots developed more slowly from callus nodules near the cut edge.

The ineffectiveness of BAP as an inducer of caulogenesis means that direct regeneration from normal leaf tissue of sugar beet cv. Primo has not yet been achieved and indirect organogenesis from callus may be the only effective means of cloning individual plants of this variety.

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