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Direct organogenesis from internodal segments of in vitro grown shoots of apple cv. Golden delicious

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ABSTRACT:

A protocol for direct organogenesis from internodal segments of *in vitro* grown shoots obtained from mature apple cv. Golden delicious trees is presented. Adventitious buds were initiated on Murashige and Skoog medium (1962) containing various combinations of benzylaminopurine (BAP) and 2,3,5-triiodobenzoic acid (TIBA). Low concentration of BAP (4.4 µM) in combination with TIBA (1 µM) gave the best percentage of regeneration. Three repeated cycles of culture and regeneration produced an increase of adventitious budding up to 23%. Although no auxin was used in the organogenic medium, callus was always obtained. The regenerated shoots were micropropagated and rooted. Cytological studies revealed that proliferating buds originated directly from the superficial layers of the internodal explants without an intermediate callus phase.

ABBREVIATIONS:

BAP=6-benzylaminopurine; IAA=indole-3-acetic acid; IBA= indole-3-butyric acid; NAA= naphthaleneacetic acid; TIBA= 2,3,5-triiodobenzoic acid.

INTRODUCTION :

Over the last decade, plant regeneration has been achieved in a number of fruit trees mostly via adventitious shoot formation (for review, see Hutchinson and Zimmerman, 1987). With apple plants, adventitious shoots have been obtained from zygotic embryos (Korban and Skirvin, 1985; Belaizi, 1989), leaves (Liu *et al.*, 1983; James *et al.*, 1988; Fasolo *et al.*, 1989), internode segments of *in vitro* grown shoots (Dufour and Zimmerman, 1986; Chevreau *et al.*, 1989), and protoplasts (Patat-Ochatt *et al.*, 1988). Although plant regeneration has been reported from leaf and internodal explants, the frequency of budding is very low, particularly from internodal segments. For example, recently Welander (1988) obtained plant regeneration from leaf segments of shoots but no organogenesis occurred on stem segments, whereas James *et al.* (1984) obtained shoots in calli derived from stem explants but with low frequency (<1%). In all cases, a study of the literature available shows that efficient budding in apple requires juvenile tissue explants. To our best knowledge, no report exists in literature about shoot regeneration from internodes of cv. Golden delicious.

In this paper we show that direct organogenesis without an intermediate callus phase can be obtained from internodal segments of apple cv. Golden delicious. Histological studies are presented showing the origin and development of buds.

MATERIAL AND METHODS:

Plant material and culture conditions of explants:

This study was done with apple (*Malus* x *domestica* Borkh.) cv. Golden delicious. Shoot cultures, which had been established 4 years earlier from mature trees, were maintained on a micropropagation medium (MM) containing basal salts,vitamins and chelated iron of Murashige and Skoog (MS, 1962) with 4.4 µM BAP and 0.49 µM IBA (Belaizi *et al.*, 1989), and were subcultured every four weeks. Internodal segments (0.5-1 cm long) were excised from shoots propagated by axillary buds. Dissection of the stem segments was done under a microscope to ensure absence of axillary buds.

The media used for internodal segment cultures were based on Murashige and Skoog medium (1962) containing 30 gl^{-1} sucrose and 7 gl^{-1} Difco Bacto agar. The media are referred to as:

- Control 1 (C1): MS + 4.4 µM BAP
- Control 2 (C2): MS + 11 µM BAP
- Control 3 (C3): MS + 22 µM BAP
- DM1 : MS + 4.4 µM BAP + 1 µM TIBA
- DM2 : MS + 11 µM BAP + 1 µM TIBA
- DM3 : MS + 22 µM BAP + 1 µM TIBA
- DM4 : MS + 22 µM BAP + 2 µM TIBA
- RM (rooting medium): 1/2 strength MS salts with 4.9 µM IBA and 15 gl^{-1} sucrose.

These levels of BAP were chosen after preliminary assays, using different hormonal combinations and concentrations, for regeneration from internodal segments.

The pH of the media was adjusted to 5.6 prior to adding agar. All components of the medium were mixed before autoclaving (120°C, 20 min).

10-15 explants were cultured per Petri dish which were sealed with parafilm. The cultures were grown at 27± 1°C under cool

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white fluorescent tubes (20-30 μE m^{-2} s^{-1}) with a 16/8 hours light/dark cycle and transferred to fresh medium every four weeks.

Histological procedures :
Internodal segments were excised over a culture period of 0 to 30 days at variable intervals and were fixed for light microscopy in order to study shoot formation. Explants were fixed in 2% glutaraldehyde in sodium cacodylate buffer, pH 7.6 at 0.1 M for 4 hours. After washing 3 times (15 min each) in sodium cacodylate buffer, they were postfixed for 1 hour in 1% osmium tetroxide in cacodylate buffer (pH 7.6). After dehydration in alcohol they were impregnated with propylene oxide and embedded in araldite. Sections (1-2 μm) cut with a glass knife (LKB ultramicrotome) were stained with gentian violet or azur methylene blue and observed under a light microscope.

Regeneration studies :
In this study, at least 48 internodal segments from proliferating shoots were used per treatment and were cultured either on DM1, DM2, DM3 or DM4 media and compared with the control. Repeated regenerations: shoot culture -----> internodes -----> shoot regeneration -----> internodes -----> shoot regeneration , were assessed over two or three cycles respectively on DM1 and DM4 fresh media. Results were scored after four weeks of culture.

As the aim was to induce shoots without a callus phase on the stem explants, only the combinations of BAP and TIBA were chosen. Moreover, our preliminary results showed that auxin such as NAA, which is widely used in apple tissue culture, induces callus even at low concentration. Therefore, it was excluded in our experiments.

Statistical analysis:
Percentage differences between regeneration rates were examined using the normal approximation to a binomial distribution. The number of shoots per explant was statistically examined using the Student test (Bresson, 1986) for significant differences.

RESULTS :

Direct organogenesis :
To explore the possibility of generating large number of buds in a more defined manner, internodal explants were inoculated onto DM1, DM2, DM3 and DM4 media. The effects of repeated regeneration of shoots and the effects of BAP and TIBA concentrations were examined. Buds or calli were initially observed after 3 weeks of culture on each medium and compared with the control. For example, Figure 1 shows the formation of adventitious buds after 3 weeks of culture in the medium DM1. After 5 to 6 weeks of culture on the same medium, the buds elongated into shoots (Fig. 2, arrow). Two types of calli were found: either white with filaments on the surface, or red and green and compact. The calli and buds always developed at the cut end of the explants.
No organogenesis was observed without TIBA although the media C1, C2 and C3 contained different concentrations of BAP (Table 1). During the first cycle of regeneration of internodal segments, organogenesis occurred in 8.5% to 12.6% of explants cultured with various combinations of BAP and TIBA with no significant difference. However, there was a significant effect of BAP on the average number of shoots obtained. The use of 22 μM of BAP in DM3 and DM4 media led to a decrease in the mean shoot regeneration as compared to lower BAP as in DM1 and DM2. Moreover, there was a significant decrease in shoot number with respect to the TIBA concentration in DM4 (2 μM) and DM3 (1 μM) media.
2 μM of TIBA was disadvantagous for shoot induction as compared to 1 μM TIBA present in DM3 medium.
Although no auxin was used in the organogenic medium, callus was always obtained but with variable percentage from 5% to 32.5% depending on the specific medium tested.

Table 1: The effect of BAP and TIBA concentrations on internodal explants of apple derived from the first cycle of culture.

Media	% of organogenic explants	Sig. level	Average nº of shoot per explant	*	% of callogenic explants	Sig. level
C1	0.0	-	0.0	-	5	n.s.
C2	0.0	-	0.0	-	0.0	+
C3	0.0	-	0.0	-	6.7	++
DM1	12.6	n.s.	2.5±1.24	ab	9.5	n.s.
DM2	8.5	n.s.	2.3±1.26	ab	12.5	n.s.
DM3	10.7	n.s.	1.5±1.20	c	10.8	n.s.
DM4	12.5	n.s.	1.0±0.00	d	32.5	+

* values with different suffix letter significantly different at p= 0.01. Significance levels: ++ p = 0.001, + p = 0.01, n.s. not significant. At least 48 explants per treatment were cultured on each of the different media. Results were scored after 4 weeks of culture.

Repeated regeneration cycles strongtly improved the percentage of regeneration (Table 2). Repeated regenerations, e.g., the second cycle of regeneration on DM1 medium, produced 16% organogenic explants and 22.9% after the third cycle of regeneration, whereas callogenesis decreased to 11.7% after an initial increase to 18% (Table 2). As with DM1 medium, repeated regeneration on DM4 also increased through cycles of culture and to about 20% for the last reculture (Table 2). However, callus formation was most frequent on the first cycle on DM4 medium (32.5%).

Table 2 : Effects of repeated organogenesis (2 or 3cycles).

Media	number of regeneration cycle	% organogenic explants	*	% callogenic explants	Sig. level
DM1	1	12.6	a	9.5	n.s.
DM1	2	16	a	18	n.s.
DM1	3	22.9	bc	11.7	n.s.
DM4	1	12.5	A	32.5	++
DM4	2	20	A	10	-

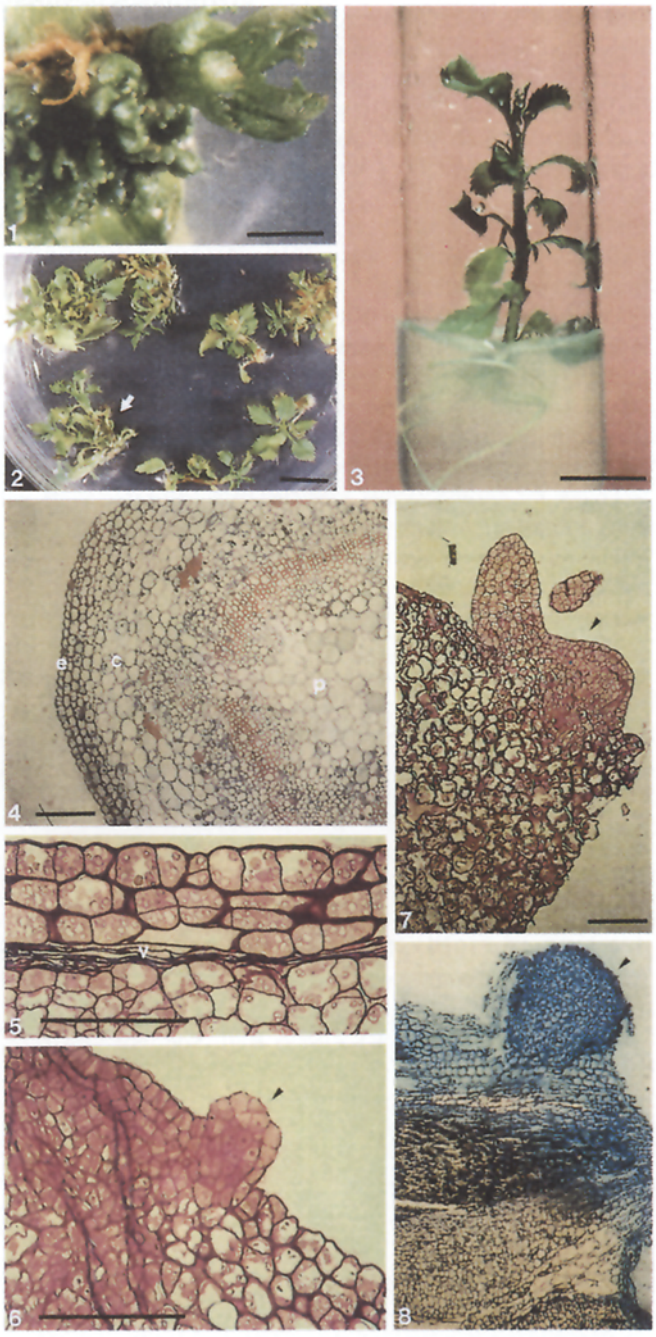
* values with different suffix letter significantly different at p= 0.05. Significance levels: ++ p = 0.001, n.s. not significant. At least 48 explants per treatment were cultured on each of the different media. Results were scored after 4 weeks of culture.

Micropropagation and rooting of adventitious buds :
Adventitious buds were transferred to the micropropagation medium (MM) for 4-5 weeks. The well developed shoots were rooted on RM in darkness for one week. Afterwards shoots were exposed to long day light conditions on an auxin-free medium. Rhizogenesis was achieved in about 80% of the explants after 4 weeks and each shoot gave 4 to 5 roots (Fig. 3).

Histological aspects :
Histological studies showed that mitotic activity was localized in epidermal and sub-epidermal cell layers near the cut surface of cultured explants (Fig. 4 and 5). The formation of the meristematic dome occurred within 2 to 4 weeks (Fig. 6). The meristematic domes developed into buds (Fig. 7) and formed adventitious shoots. Buds emerged near the distal part of the explant, hence regeneration is polarized. Callus emerged at the distal (Fig. 8) and/or at the proximal part of the internode.

DISCUSSION :

Our study achieved direct budding from internodal explants of apple cv. Golden delicious. Hormone concentrations and repeated regeneration cycles proved to be important factors affecting the rate of regeneration. Generally in apple both cytokinin and auxin are necessary for regeneration from leaves (James *et al.*, 1984, 1988; Welander, 1988) or from stem internodes (James *et al.*, 1984). In our experiments, no auxin was used and direct regeneration occurred on non callogenic explants, using TIBA and BAP. Morphogenesis occurred only at the cut end of the explant.
TIBA is a specific inhibitor of IAA transport (Chee and Cantliffe, 1989) acting at the efflux site of cells. Moreover, it inhibits auxin transmembrane transport (Rubery, 1987). Thus TIBA may block the transport of endogenous auxins in the cultured internodal explants and may cause confined accumulation of auxin. It is also possible that high endogenous auxin levels might be present in the internodal explants. Hence, TIBA could counteract the auxin inhibition of regeneration as seen with *Betula* stem segments cultures (Mc Cown, 1989). The action of TIBA, combined with BAP, would permit the establishment of an optimal auxin/cytokinin balance, which could be favorable to the expression of meristematic characters in the internodal explant. Further investigations are required to elucidate the role of TIBA in inducing bud formation in internodal explants of apple. However, earlier studies have shown that TIBA induces considerably the organogenic capacity of petiole explants of sugarbeet (Detrez *et al.*, 1988).
In our study, buds are obtained directly from internodes without any auxin, and without any dark treatment, whereas James *et al.* (1984) used both these factors to obtain buds.Indeed, light has been reported to be inhibitory from leaves or parts of apple seeds (Kouider *et al.*, 1984; Welander, 1988).In the presence of light, to our best knowledge, this phenomenon of 'improved regeneration from internodes after several cycles of culture' has never been reported in apple tissue culture and remains unexplained. Moreover, the number of reactive explants *i.e.*, regeneration frequency was significantly improved by repeated cycles of culture on DM1 medium. Similarly, James *et al.* (1988) observed a significant increase in mean shoot number in leaf discs derived from twice regenerated shoots in darkness, although the number of discs regenerating was not affected.



Figures. 1-8 : 1.) Adventitious buds from internodal segments after 3 weeks of culture, scale bar = 5 mm. 2.) Numerous proliferating shoots on internodal segments after 5 weeks of culture, scale bar = 10 mm. 3.) Rooting of the regenerated shoot in the presence of IBA (4.9 µM) after 4 weeks of culture, scale bar = 10 mm. 4.) Cross-section of an internodal segment at day 0 culture (control), e: epidermis, c: cortex, p: pith, scale bar = 200 µm. 5.) Section showing cell division activities in the epidermal and sub-epidermal cell layers, after 5 days of culture, v: vascular bundle, scale bar = 125 µm. 6.) Formation of a meristematic dome (arrow) after 2 weeks of culture, scale bar = 250 µm. 7.) Aspect of adventitious bud with leaf primordia (arrow) after 4 weeks of culture, scale bar = 500 µm. 8.) Callus formation (arrow) from an internodal segment after 4 weeks of culture, scale bar = 100 µm.

Differences between the internodes that had produced shoots and those that did not could be explained by the position of the internode on the shoot, although this factor has not yet been tested. Recently, Fasolo *et al.* (1989) reported greater regenerative capacity from young leaves near the tip in certain apple cultivars. Whether such a gradient exists in internodes is not known.

Callus was only obtained on non-organogenic explants and no indirect organogenesis was observed in these calli after microscopical examination.

In conclusion, we believe that the *in vitro* regeneration system described here represents a new technique to induce adventitious budding. Recently, this method has also been found to be effective with the genotypes M111 and Mutsu . However further investigation to optimize the rate of regeneration seems necessary. In the future, this system could provide a very useful technique for *Agrobacterium* mediated gene transfer in apple and will complement the earlier leaf explant transformation technique reported by James *et al.* (1989).

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