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# Adventitious shoot formation on excised leaves of in vitro grown shoots of apple cultivars

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**Abstract**. Leaves taken from micropropagated shoots of several apple (*Malus domestica* Borkh.) cultivars were cultured in vitro on Linsmaier & Skoog (LS) medium or the rice anther culture medium of Chu et al. (N6) containing various concentrations of either benzyladenine (BA) or thidiazuron (TDZ) plus naphthaleneacetic acid (NAA). Of the TDZ concentrations tested, 10 $\mu$M was most effective and it was equivalent to, or better than, 22 $\mu$M BA for both the percentage of leaves regenerating shoots and number of shoots formed per regenerating leaf in almost every experiment. Lower concentrations of NAA (1.1 and 5.4 $\mu$M) gave best results with both BA and TDZ. N6 medium gave consistently better results than LS. Lowering total salt concentration or total N concentration of LS to that of N6 did not improve the response nor did changing the $NO_3$:$NH_4$ ratio. The 3–4 leaves on the most distal part of the shoot were most responsive and tended to form the most adventitious shoots. Placing the leaf cultures in the dark for the first 2–3 weeks of the culture period produced the best results. Optimum results were obtained by culturing leaves from the distal part of the shoot in the dark for 2 weeks on N6 medium containing 10 $\mu$M TDZ and 1.1 or 5.4 $\mu$M NAA, then moving the cultures to 16 h daylight at a photon flux of 60 $\mu$mol $s^{-1}$ $m^{-2}$.

## Introduction

Regeneration of plants from adventitious shoots is necessary for application of gene transfer technology and for screening plants for somaclonal variation. The latter purpose would be of particular interest with apples where conventional breeding programs have had relatively little impact upon commercial apple production. Regeneration of adventitious shoots has been reported from apple cotyledons [7, 8, 9, 12, 13], embryo axes [6], seedling leaves and hypocotyls [11], callus initiated from both seedling leaves [11] and roots of intact microplants of M.25 rootstock [5], and from leaves or leaf disks obtained from in vitro propagated shoots [2, 3, 4, 16, 18, 19] or

greenhouse-grown plants [12] of mature clones.[1] The media used for these studies contained benzyladenine (BA) plus an auxin and, in some cases [6, 7, 8, 9], coconut milk. In addition, a cytokinin-like chemical, thidiazuron (*N*-phenyl-*N'*-1,2,3-thiadiazol-5-ylurea), was shown to stimulate production of adventitious shoots of 'Gala' apple in vitro [17].

We report here regeneration of adventitious shoots from leaf explants of several apple cultivars using either BA or thidiazuron (TDZ) in the medium.

## Materials and methods

Shoots were excised aseptically from in vitro grown cultures, originally established 3 to 6 years earlier, of the apple cultivars 'McIntosh', 'Paladino Spur McIntosh', 'Triple Red Delicious' and 'Gala' and were collected in a jar of sterile distilled water. One shoot was used to provide leaves for each Petri dish. Each unfurled leaf longer than 5 mm was excised from the shoot, the petiole was removed and, in the middle part of the leaf, three transverse cuts were made through the midrib at 1 mm intervals without severing the leaf completely. Each leaf was then placed with the adaxial side touching the medium.

The media used were Linsmaier & Skoog (LS) [10] and the N6 medium developed for rice anther culture [1]. Composition of the basal media and modifications of them that were tested are listed in Table 1. Other components of all media were LS micronutrients, 0.56 mM myo-inositol, 3 $\mu$M thiamine-HCl, 87.6 mM sucrose and 0.7% Difco Bacto agar. Growth regulators used were either 22 $\mu$M BA or 0.1, 1, 5, 10, or 50 $\mu$M TDZ, both in combination with 1.1, 5.4, or 10.7 $\mu$M naphthaleneacetic acid (NAA). The pH of the medium was adjusted to 5.2 prior to adding agar. All media were autoclaved for 15 min at 121 °C and 1.1 kg cm$^{-2}$ in flasks and then dispensed aseptically, 25 ml per 100 × 15 mm sterile plastic Petri dish. After leaves were explanted on the medium, the dishes were sealed with parafilm. The cultures were placed in the dark at 25 °C for the entire culture period in most experiments. When moved to the light, the conditions were 25 ± 2 °C with a 16 h photoperiod provided by warm white fluorescent tubes at a photon flux of about 60 $\mu$mol s$^{-1}$ m$^{-2}$. Leaves were transferred to fresh medium every month.

Factors that were tested in these experiments were cytokinin type and concentration, auxin concentration, medium composition (salt mixture,

[1] Although references 2, 4, 12, 16, and 19 are not widely available, they are cited here to indicate relevant research underway contemporaneously with that reported here.

*Table 1.* Major salts used in media (mM), total potassium and nitrogen content (mM), and ratio of $NO_3^-$ to $NH_4^+$.

| | LS | LS–A | LS–B | N6 | N6–A | N6–B | N6–C |
|---|---|---|---|---|---|---|---|
| $NH_4NO_3$ | 20.6 | 11.95 | 11.95 | – | – | 2.0 | – |
| $(NH_4)_2SO_4$ | – | – | – | 3.5 | 5.85 | 0.95 | – |
| $KNO_3$ | 18.8 | 10.90 | 10.90 | 28.0 | 23.3 | 28.0 | 30.94 |
| $CaCl_2 . 2H_2O$ | 3.0 | 1.74 | 1.74 | 1.13 | 1.13 | 0.58 | – |
| $MgSO_4 . 7H_2O$ | 1.5 | 0.87 | 0.87 | 0.75 | 0.75 | 0.75 | – |
| $KH_2PO_4$ | 1.25 | 0.72 | 0.72 | 2.94 | 2.94 | 2.94 | – |
| $K_2SO_4$ | – | – | 9.66 | – | 2.35 | – | – |
| $Ca(NO_3)_2 . 4H_2O$ | – | – | – | – | – | 0.55 | 1.13 |
| $Mg(NO_3)_2 . 6H_2O$ | – | – | – | – | – | – | 0.75 |
| $NaH_2PO_4$ | – | – | – | – | – | – | 2.94 |
| Total K (mM) | 20 | 11.6 | 30.9 | 30.9 | 30.9 | 30.9 | 30.9 |
| Total N (mM) | 60 | 34.8 | 34.8 | 35.0 | 35.0 | 35.0 | 34.7 |
| $NO_3$:$NH_4$ | 2:1 | 2:1 | 2:1 | 4:1 | 2:1 | 8:1 | all $NO_3$ |

nitrogen concentration, nitrate-ammonium ratio), number of days between transfer of stock cultures and leaf collection, number of days that cultures remained in the dark, leaf position on the stem and effect of cold storage of the source cultures. Four replicate Petri dishes, each containing five leaves, were used for all experiments except the ones for testing the effect of leaf position. For the latter experiments, the uppermost 7 leaves from each of 9 shoots were used for each of the four cytokinin treatments tested.

The number of leaves forming shoots and the number of shoots formed per leaf were recorded at approximately one-month intervals for 3 months. For this purpose, adventitious buds were counted as shoots. Data were analysed using the General Linear Models Procedure of SAS [14]. Data on number of leaves forming shoots were subjected to arcsin transformation for proportions [15] before analysis and were transformed back to percentages for presentation in tables.

## Results

In all experiments, shoot formation occurred within one month after the leaves were explanted on the medium (Fig. 1). Shoot formation continued through the third month, although the increase after the second month was generally small. Adventitious buds formed mainly on or in callus (Fig. 2) that developed on the distal side of the cuts across the midrib of the leaves, but sometimes appeared to arise directly from leaf tissue (Fig. 3).

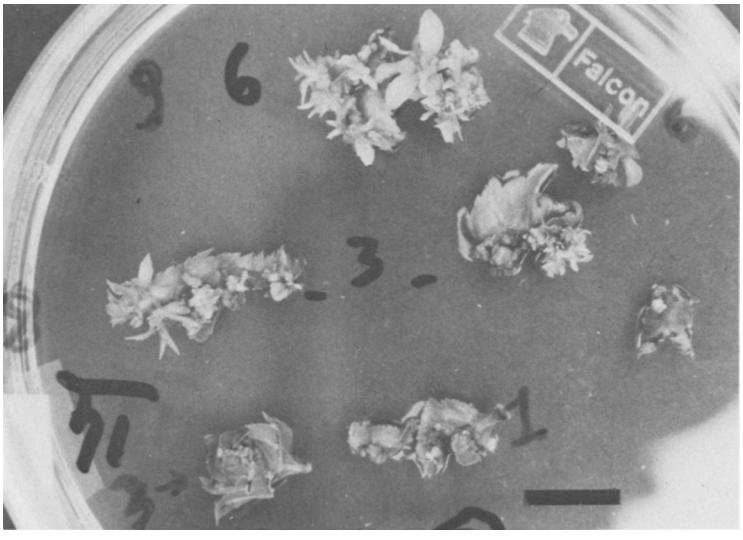


*Fig. 1.* Adventitious shoots on leaves of 'Triple Red Delicious' apple after 2 weeks in the dark followed by 2 weeks in light on N6 medium containing 22 $\mu$M BA and 1.1 $\mu$M NAA (bar = 1 cm).

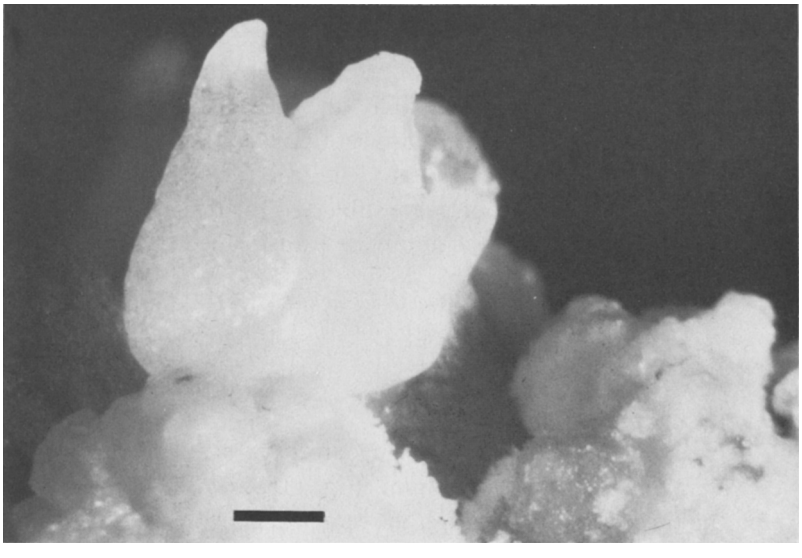

*Fig. 2.* Adventitious bud growing from callus on leaf of 'Delicious' apple after 3 months in the dark on N6 medium containing 10 $\mu$M TDZ and 5.4 $\mu$M NAA (bar = 0.5 mm).

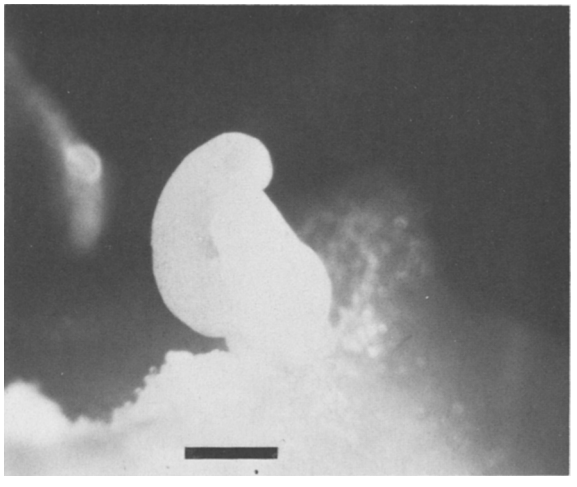

*Fig. 3.* Adventitious bud growing from leaf of 'McIntosh' after 3 months in the dark on N6 medium containing 10 $\mu$M TDZ and 5.4 $\mu$M NAA (bar = 0.5 mm).

The percentage of cultures forming adventitious shoots and the number of shoots formed per regenerating leaf were highest with 22 $\mu$M BA and 10 $\mu$M TDZ for 'McIntosh' and 'Paladino Spur McIntosh' (Tables 2 and 3). For 'Triple Red Delicious', 10 $\mu$M TDZ stimulated the most leaves to produce shoots and 1 and 10 $\mu$M TDZ yielded the highest number of shoots per regenerating leaf. For all cultivars, 0.1 $\mu$M TDZ had little if any effect. As a result, only 10 $\mu$M TDZ and 22 $\mu$M BA were used in later experiments.

Little difference in response to the two lower concentrations (1.1 and 5.4 $\mu$M) NAA were found in general, but 10.7 $\mu$M NAA usually reduced the number of leaves responding and the number of shoots produced. Interaction between NAA and cytokinin was generally not observed, but when it occurred, it was usually in data taken after 1 month. In data taken after 3 months, the interaction between auxin and cytokinin was significant ($P = 0.01\%$) for the number of shoots regenerated on 'McIntosh' leaves that were excised 20 days after subculturing (see main effects in Table 3). This significant interaction resulted from the almost total lack of response to NAA by leaves growing on medium containing 0.1 $\mu$M TDZ, whereas leaves on the other three cytokinin treatments showed increased response as NAA increased from 1.1 to 5.4 $\mu$M, and then a large decrease with further increase of NAA to 10.7 $\mu$M. In contrast, the significant interaction for number of shoots produced on 'McIntosh' leaves 3 months after excision was characterized by a different response pattern to NAA for each cytokinin treatment (see main effects in Table 2).

*Table 2.* Main effects of auxin concentration and cytokinin type and concentration on regeneration of adventitious shoots from apple cultivar leaves cultured in the dark on N6 medium and evaluated after 1, 2 and 3 months.[1]

| Auxin or cytokinin | Concentration ($\mu$M) | McIntosh | | | Triple Red Delicious | | |
|---|---|---|---|---|---|---|---|
| | | 1 (months) | 2 | 3 | 1 (months) | 2 | 3 |
| *Leaves regenerating (%)* | | | | | | | |
| NAA | 1.1 | 2 a[2] | 28 a | 41 b | 50 a | 59 a | 61 a |
| | 5.4 | 3 a | 42 a | 66 a | 25 ab | 39 a | 42 ab |
| | 10.7 | 0 b | 43 a | 60 a | 4 b | 9 b | 16 b |
| | | *[3] | ns | * | ** | ** | ns |
| BA | 22 | 11 a | 82 a | 92 a | 9 b | 11 b | 12 b |
| TDZ | 0.1 | 0 b | 2 d | 9 d | –[4] | – | – |
| | 1 | 0 b | 26 c | 43 c | 12 b | 24 b | 32 b |
| | 10 | 1 b | 54 b | 77 b | 57 a | 71 a | 76 a |
| | | *** | *** | *** | ** | ** | *** |
| *Number of shoots per regenerating leaf* | | | | | | | |
| NAA | 1.1 | 1.0 b | 2.2 a | 2.7 a | 3.5 c | 3.8 b | 4.2 a |
| | 5.4 | 1.6 a | 2.8 a | 3.6 a | 5.0 b | 4.5 ab | 4.0 a |
| | 10.7 | – | 2.2 a | 2.7 a | 6.5 a | 6.2 a | 5.4 a |
| | | ns | ns | ** | ** | ns | ns |
| BA | 22 | 1.4 a | 3.8 a | 5.0 a | 2.0 b | 2.2 b | 2.3 b |
| TDZ | 0.1 | – | 1.2 b | 1.5 b | – | – | – |
| | 1 | – | 1.4 b | 2.0 b | 7.1 a | 6.1 a | 5.2 a |
| | 10 | 1.0 a | 2.3 b | 3.0 b | 5.7 a | 6.1 a | 6.1 a |
| | | ns | *** | ** | *** | *** | ** |

[1]Means for auxin include all cytokinin treatments; means for cytokinin include all auxin treatments.
[2]Mean separation in groups within columns by Duncan's Multiple Range Test, 5% level.
[3]*F* test: ns, *, **, *** – not significant, significant at 5%, 1% or 0.1% level, respectively.
[4]No response; data not included in analysis.

Responses recorded at the end of 3 months showed relatively little increment over those recorded at the end of 2 months (Table 2). In later experiments (e.g., Tables 4 and 6), a greater response at the end of 1 month was found than is shown in Table 2.

Little difference was noted in response of leaves collected from mother cultures 20 or 40 days after subculture (Table 3). Similar results obtained

*Table 3.* Main effects of auxin concentration and cytokinin type and concentration on regeneration of adventitious shoots from apple cultivar leaves collected 20 or 40 days after subculture and evaluated after 3 months culture in the dark on N6 medium.[1]

| Auxin or cytokin | Concentration (μM) | McIntosh | | Paladino Spur McIntosh | |
|---|---|---|---|---|---|
| | | 20 (days) | 40 | 20 (days) | 40 |
| *Leaves regenerating (%)* | | | | | |
| NAA | 1.1 | 90 a[2] | 65 a | 65 a | 72 ab |
| | 5.4 | 88 a | 70 a | 77 a | 79 a |
| | 10.7 | 78 a | 22 b | 38 b | 57 b |
| | | ns[3] | *** | *** | ns |
| BA | 22 | 99 a | 84 a | 86 a | 96 a |
| TDZ | 0.1 | 10 b | 0 c | 4 c | 4 c |
| | 1 | 95 a | 44 b | 53 b | 65 b |
| | 10 | 99 a | 93 a | 95 a | 99 a |
| | | *** | *** | *** | *** |
| *Number of shoots per regenerating leaf* | | | | | |
| NAA | 1.1 | 6.0 b | 4.8 a | 4.2 a | 4.5 ab |
| | 5.4 | 8.0 a | 5.1 a | 4.2 a | 5.2 a |
| | 10.7 | 3.3 c | 2.2 b | 2.5 b | 3.5 b |
| | | *** | * | ns | ns |
| BA | 22 | 6.5 b | 4.2 ab | 3.8 b | 6.1 a |
| TDZ | 0.1 | 1.4 d | – | 1.0 c | 1.2 b |
| | 1 | 4.4 c | 2.7 b | 2.1 bc | 2.2 b |
| | 10 | 9.3 a | 4.9 a | 6.0 a | 6.3 a |
| | | *** | ns | *** | *** |

[1]Means for auxin include all cytokinin treatments; means for cytokinin include all auxin treatments.
[2]Mean separation in groups within columns by Duncan's Multiple Range Test, 5% level.
[3]*F* test: ns, *, **, *** – not significant, significant at 5%, 1% or 0.1% level, respectively.

with 'Paladino Spur McIntosh' leaves cultured 30 days following the preceding shoot subculture confirmed this (data not shown).

Shoots produced on leaves grown on 22 μM BA elongated whereas those produced on leaves grown on 10 μM TDZ were very compact with short internodes and small leaves (Fig. 4).

Comparison of the N6 and LS media clearly showed that N6 was superior (Table 4). When these media were modified, the lower nitrogen level produced better results with 'Triple Red Delicious' but had little effect on 'McIntosh' (Table 4). The form of nitrogen had a much greater effect on regeneration than did nitrogen level, with the higher proportion of nitrate

*Table 4.* Comparison of medium composition and cytokinin on regeneration of adventitious shoots from apple cultivar leaves after 1, 2 and 3 months in the dark.[1]

| Comparison | McIntosh | | | Triple Red Delicious | | |
|---|---|---|---|---|---|---|
| | 1 (months) | 2 | 3 | 1 (months) | 2 | 3 |
| *Leaves regenerating (%)* | | | | | | |
| LS | 55 | 89 | 95 | 3 | 21 | 56 |
| N6 | 92 | 96 | 99 | 80 | 92 | 97 |
| | ***[2] | ns | ns | *** | *** | ** |
| 35 mM N | 55 | 78 | 88 | 32 | 62 | 72 |
| 60 mM N | 55 | 89 | 95 | 3 | 21 | 56 |
| | ns | ns | ns | *** | ** | * |
| $NO_3$:$NH_4$ 4:1 | 92 | 96 | 99 | 80 | 92 | 97 |
| 2:1 | 43 | 76 | 86 | 6 | 39 | 64 |
| | ** | * | ns | *** | *** | *** |
| BA 22 $\mu$M | 47 | 74 | 88 | 18 | 37 | 61 |
| TDZ 10 $\mu$M | 66 | 90 | 95 | 22 | 69 | 86 |
| | * | ns | ns | ns | * | * |
| *Number of shoots per regenerating leaf* | | | | | | |
| LS | 1.5 | 2.4 | 4.6 | – | 1.6 | 1.6 |
| N6 | 3.5 | 4.4 | 6.6 | – | 3.8 | 5.9 |
| | *** | *** | *** | | *** | *** |
| 35 mM N | 2.3 | 2.8 | 4.3 | – | 2.5 | 3.8 |
| 60 mM N | 1.5 | 2.4 | 4.6 | – | 1.6 | 1.6 |
| | ** | ns | ns | | ns | ** |
| $NO_3$:$NH_4$ 4:1 | 3.5 | 4.4 | 6.6 | 2.7 | 3.8 | 5.9 |
| 2:1 | 1.6 | 2.1 | 3.6 | 1.4 | 1.5 | 2.4 |
| | *** | *** | *** | ** | *** | *** |
| BA 22 $\mu$M | 2.0 | 2.6 | 3.6 | 2.0 | 2.1 | 2.3 |
| TDZ 10 $\mu$M | 2.2 | 2.8 | 5.1 | 1.9 | 2.5 | 4.2 |
| | ns | ns | ** | ns | ns | ** |

[1]Experimental design was a factorial with four media (LS, LS–A, N6, N6–A; see Table 1) and two cytokinin treatments; following GLM analysis [8], sum of squares for media treatments was partitioned by single degree of freedom contrasts.

[2]ns, *, **, *** – not significant, significant at 5%, 1% or 0.1%, respectively.

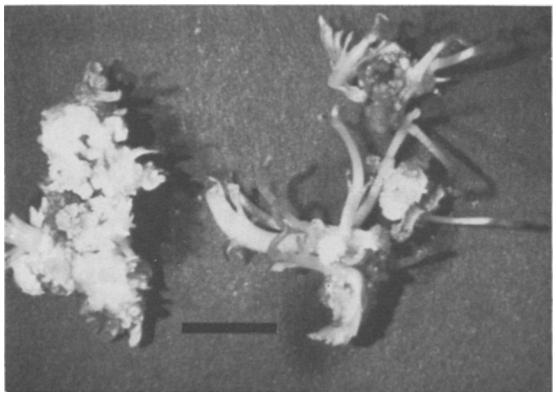

*Fig. 4.* Adventitious shoot production on leaves of 'Triple Red Delicious' cultured for 3 months in the dark on N6 medium containing 10 $\mu$M TDZ (left) or 22 $\mu$M BA (right) (bar = 1 cm).

yielding better results with both cultivars (Table 4). The response of both cultivars to 10 $\mu$M TDZ was always equal to or greater than the response to 22 $\mu$M BA (Table 4).

Based on the results obtained in the previous experiments, the $NO_3$:$NH_4$ ratio was examined further (Table 5). Raising nitrate level to 89% of the total N gave no better results than 80%, whereas raising it to 100% completely eliminated adventitious shoot regeneration.

*Table 5.* Effect of different $NO_3$:$NH_4$ ratios in the medium on regeneration of adventitious shoots from leaves of apple cultivars after 2 months in the dark.[1]

| Medium | $NO_3$:$NH_4$ | McIntosh | Triple Red Delicious |
|---|---|---|---|
| *Leaves regenerating (%)* | | | |
| N6C | all $NO_3$ | 0 | 0 |
| N6B | 8:1 | 93 a[2] | 87 a |
| N6 | 4:1 | 97 a | 95 a |
| LSB | 2:1 | 64 b | 29 b |
| *Number of shoots per regenerating leaf* | | | |
| N6C | all $NO_3$ | 0 | 0 |
| N6B | 8:1 | 5.8 a | 2.9 ab |
| N6 | 4:1 | 6.2 a | 3.6 a |
| LSB | 2:1 | 1.8 b | 1.6 b |

[1]Data are means of two cytokinin treatments, 22 $\mu$M BA and 10 $\mu$M thidiazuron; data for N6C are not included in statistical analysis.

[2]Mean separation within columns by Duncan's Multiple Range Test, 5% level.

*Table 6.* Leaf position effect on regeneration of adventitious shoots from apple cultivar leaves after 1, 2 or 3 months in the dark[1]

| Leaf position[2] | McIntosh | | | Triple Red Delicious | | |
|---|---|---|---|---|---|---|
| | 1 (months) | 2 | 3 | 1 (months) | 2 | 3 |
| *Leaves regenerating (%)* | | | | | | |
| 1 | 75 ab[3] | 96 abc | 100 a | 49 ab | 93 a | 96 a |
| 2 | 84 a | 100 a | 100 a | 75 a | 91 a | 93 ab |
| 3 | 66 abc | 98 ab | 100 a | 65 ab | 91 a | 91 ab |
| 4 | 52 abc | 96 abc | 99 ab | 48 ab | 65 ab | 78 abc |
| 5 | 38 abc | 75 bcd | 88 bc | 40 ab | 58 ab | 69 abc |
| 6 | 25 bc | 66 cd | 81 cd | 22 b | 38 b | 52 bc |
| 7 | 16 c | 35 d | 59 d | 25 b | 59 ab | 65 bc |
| *Number of shoots per regenerating leaf* | | | | | | |
| 1 | 3.3 a | 4.2 a | 5.4 ab | 2.1 ab | 2.7 ab | 4.8 a |
| 2 | 3.4 a | 4.1 a | 5.7 a | 2.3 ab | 3.0 ab | 4.5 a |
| 3 | 2.9 a | 3.4 ab | 4.7 abc | 2.5 a | 3.5 a | 5.2 a |
| 4 | 3.4 a | 3.1 ab | 3.9 abcd | 1.8 ab | 2.4 ab | 3.4 ab |
| 5 | 3.1 a | 2.8 ab | 3.0 cd | 1.3 b | 1.9 b | 2.6 b |
| 6 | 3.2 a | 3.6 ab | 3.8 bcd | 2.0 ab | 2.0 b | 2.4 b |
| 7 | 3.1 a | 2.4 b | 2.5 d | 1.4 ab | 1.9 b | 2.6 b |

[1]Data are means for three cytokinin treatments: 22 $\mu$M BA, and 5 and 10 $\mu$M TDZ; 50 $\mu$M TDZ was also tested in this experiment but no adventitious shoots were formed.
[2]Leaf 1 is nearest the shoot apex and exceeded 5 mm in length at time of excision; 27 leaves for each position.
[3]Mean separation in columns by Duncan's Multiple Range Test, 5% level.

Position of the leaf on the shoot also affects adventitious shoot regeneration (Table 6). A gradient in response exists from the tip to the base of the shoot with the distal leaves being more responsive and tending also to form the most shoots per regenerating leaf. In these experiments, leaves from each position were grown on medium containing 22 $\mu$M BA, or 5, 10 or 50 $\mu$M TDZ. No adventitious shoots formed on 50 $\mu$M TDZ, while 22 $\mu$M BA and 5 $\mu$M TDZ were equal to or less effective than 10 $\mu$M TDZ (data not shown).

The length of time that the leaf cultures were kept in the dark before transfer to the light also affected regeneration of adventitious shoots (Table 7). Two weeks of dark treatment were sufficient to give maximum response.

Leaves taken from shoot cultures that had been stored at least 30 days at 2 °C were no more likely to form adventitious shoots on N6 medium with 22 $\mu$M BA than leaves from shoot cultures maintained at 25 °C in a growth room. However, responsive leaves from cold-stored cultures of 'Gala'

*Table 7.* Effect of time in dark on the regeneration of adventitious shoots from leaves of apple cultivars grown on N6 medium with 10 $\mu$M thidiazuron for 7 weeks.

| Time (weeks) | | McIntosh | Triple Red Delicious |
|---|---|---|---|
| Dark | Light | | |
| *Leaves regenerating (%)* | | | |
| 0 | 7 | 0 c[1] | 0 b |
| 1 | 6 | 23 b | 15 b |
| 2 | 5 | 88 a | 91 a |
| 3 | 4 | 41 b | 59 a |
| 7 | 0 | 69 ab | 96 a |
| *Number of shoots per regenerating leaf* | | | |
| 0 | 7 | 0[2] | 0[2] |
| 1 | 6 | 1.3 a | 1.5 c |
| 2 | 5 | 2.1 a | 4.7 ab |
| 3 | 4 | 2.1 a | 5.4 a |
| 7 | 0 | 1.9 a | 2.5 bc |

[1]Mean separation within columns by Duncan's Multiple Range Test, 5% level.
[2]Not included in statistical analysis.

formed significantly more shoots after 3 months than did the control leaves (14 vs. 9). Leaves from cold-stored shoots of 'McIntosh' showed a similar tendency whereas those of 'Paladino Spur McIntosh' did not differ from the controls.

## Discussion

The data presented above demonstrate clearly that adventitious shoot regeneration is possible from leaves of all the apple cultivars tested: 'McIntosh', 'Paladino Spur McIntosh', 'Triple Red Delicious' and 'Gala'. Furthermore, the adventitious shoots can be excised from the leaves and cultured on proliferation medium where they grow in the same manner as do shoots derived from axillary buds.

Thidiazuron appears to be more effective than BA for inducing adventitious shoots to form on excised leaves from micropropagated shoots, with 10 $\mu$M yielding the best results. These results confirm the earlier speculation [17] that thidiazuron stimulates production of adventitious shoots. Adventitious shoots induced by TDZ are compact with short internodes and this growth habit can persist through several subcultures even after such shoots are transferred to a proliferation medium containing 4.4 $\mu$M BA. Eventually

normal elongation occurs and shoots can be rooted as reported previously [17].

As found also by Welander [19], the N6 medium was superior to LS for regeneration of adventitious shoots. The N6 medium differs from LS in several respects, having lower total salt content, lower N, Ca, and Mg content, a lower proportion of $NH_4$-N, and higher concentrations of K and P. Changing the amount of N in LS or the $NO_3$:$NH_4$ ratio in N6 did not increase regeneration of adventitious shoots and usually decreased it (Tables 4 and 5). Liu [12] reported that supplementing LS medium with 1000 mg $l^{-1}$ $KNO_3$ increased adventitious shoot regeneration for leaf disks of greenhouse-grown 'Empire' apple. Thus it might be of interest to examine further the $KNO_3$ concentration as well as other components of the N6 medium to see if it can be improved for adventitious shoot regeneration.

Position of the leaf on the shoot influenced the capacity to regenerate adventitious shoots (Table 6). More recently formed leaves near the tip of the shoot had greater regenerative capacity.

Although adventitious shoots formed in continuous darkness, only two weeks of darkness proved to be sufficient (Table 7). This agrees with the reports of Welander [19] and Liu [12]. Shoots grown in continuous darkness were etiolated. Such shoots were very elongated when grown on 22 $\mu$M BA, but not when grown on TDZ (Fig. 4).

Use of leaves from in vitro propagated shoots eliminates the need for disinfestation procedures with the possibilities of tissue damage and contamination when using leaves or leaf disks from greenhouse-grown plants as reported by Liu [12]. Use of whole leaves, even with the midrib cut as done here, is simpler than collecting leaf disks from in vitro grown leaves [3, 4] or cutting the leaf blades into 1 mm wide strips [18, 19].

To summarize, adventitious shoots can be produced by culturing the distal 3–4 leaves from in vitro produced shoots of apple on N6 medium containing 10 $\mu$M TDZ or 22 $\mu$M BA. Cultures should be grown in the dark for 2 weeks before transferring to light at a photon flux of about 60 $\mu$mol $s^{-1}$ $m^{-2}$. This technique has also worked with several other apple cultivars in experiments outside the scope of the studies reported here.

## References

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