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Microtuberization of layered shoots and nodal cuttings of potato: The influence of growth regulators and incubation periods

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Abstract

A protocol is presented for the rapid induction of microtubers on micropropagated, layered potato shoots of 'Kennebec', 'Russet Burbank' and 'Superior' in medium devoid of growth regulators. Layered shoots microtuberized more rapidly and produced significantly larger microtubers compared with nodal cuttings. The addition of coumarin or (2-chloroethyl)-trimethylammonium chloride and benzyladenine to microtuberization medium, either had no effect or significantly reduced microtuber weight per shoots compared with medium containing only 80 g $\times$ l^{-1} sucrose and minimally affected the number of microtubers per shoot. Increasing the incubation period from 28 to 56 days did not affect the number but significantly increased the weight of microtubers per shoot and substantially increased the proportion, up to 20%, of microtubers heavier than 1 gram.

Abbreviations: Ba – benzyladenine, ccc – (2-chloroethyl) trimethylammonium chloride, coumarin – 2*h*-1-benzopyran-2-one, ga_3 – gibberellic acid

Introduction

The first report of in vitro tuberization was published by Barker (1953) who used etiolated sprouts to induce tuberization in medium containing 80 g $\times$ l^{-1} sucrose. Since then the utilization of growth regulating agents to favour tuberization in vitro (microtuberization) has been the object of intensive investigation. Among the substances used to induce microtubers, coumarin, CCC and cytokinins have received most attention (Wattimena 1983; Wang & Hu 1985; Chandra et al. 1988).

Cytokinins and coumarin are believe to have strong promotive effects on tuberization, and to constitute part of the tuberization stimulus, either alone or in combination with other substance(s) (Palmer & Smith 1969, 1970; Forsline & Langille 1976; Stallknecht & Farnsworth 1982a, 1982b; Pelacho & Mingo-Castel 1991). However, growth regulators failed to induce tuberization when the sucrose supply was inadequate (Harmey et al. 1966). Sucrose may be the only compound necessary for induction of microtuberization (Gregory 1956; Ewing 1985, 1990).

A reliable microtuber production method on medium free of any growth regulating agent was reported by Garner & Blake (1989). Nodal cuttings were grown on medium containing MS basal salts (Murashige & Skoog 1962) with 80 g $\times$ l^{-1} sucrose, and incubated first under a 16 h and then an 8 h photoperiod. After 17 wks of incubation each cutting had produced approximately 1 microtuber weighing < 200 mg.

Increasing the productivity of microtuberization systems while reducing medium complexity is a desirable objective. To realize this goal experiments were conducted to:

- compare microtuberization productivity of layered shoots with nodal cuttings
- evaluate the efficiency of coumarin, CCC and BA as promoters of microtuberization, and

– evaluate the effect of the duration of incubation period on microtuber yield.

Materials and methods

Multiplication of in vitro material

Nodal cuttings of 'Kennebec', 'Superior' and 'Russet Burbank' were grown in 25 × 150 mm pyrex glass culture tubes on 15 ml of modified MS basal salt solution supplemented with 0.4 mg l^{-1} thiamine-HCl, 2 mg l^{-1} Ca-pantothenate, 100 mg l^{-1} inositol, 30 g l^{-1} sucrose, 7 g l^{-1} agar (Anachemia, Lachine, Quebec). The pH was adjusted to 5.7 prior to autoclaving at 121 °C for 15 min. Cultures were grown at 22 ± 2 °C under 85 µmol m^{-2} s^{-1} cool white fluorescent illumination and 16 h photoperiod and were subcultured every 4 weeks.

Microtuber production using layered shoots

Microtubers were induced by a modified two-step procedure (Estrada et al. 1986; Meulemans et al. 1986). In the first step, 3 root-severed plantlets (shoots) with 6 nodes each were layered in 50 ml of liquid propagation medium containing modified MS basal salt solution supplemented with thiamine-HCl, Ca-pantothenate and inositol, as previously described, and 0.4 mg l^{-1} GA_3, 0.5 mg l^{-1} BA, 20 g l^{-1} sucrose, in 400 ml plastic containers (Better Plastics, Kissamee, Fla.). This first stage, which promotes vegetative growth prior to microtuberization, significantly increased microtuber weight and number per shoot compared with shoots placed directly into microtuberization medium. Cultures were incubated at 22 ± 2 °C under 85 µmol m^{-2} s^{-1} cool white fluorescent illumination and 16-h photoperiod. After 4 weeks the residual medium was drained off and replaced by 50 ml of microtuberization media containing modified MS basal salt solution supplemented with thiamine-HCl, Ca-pantothenate, inositol, as previously described, and either

– 50 mg l^{-1} coumarin and 80 g l^{-1} sucrose,
– 500 mg l^{-1} CCC, 5.0 mg l^{-1} BA (CCC-BA) and 80 g l^{-1} sucrose, or
– a control medium containing 80 g l^{-1} sucrose but no growth regulator.

Layered shoots were incubated at 15 ± 2 °C under 50 µmol m^{-2} s^{-1} cool white fluorescent illumination and 8-h photoperiod.

Microtuber production using modal cuttings

Three in vitro plantlets with 6 nodes each were sectioned into single node cuttings (the shoot apex was discarded) and induced to tuberize in 50 ml of microtuberization medium (media a-c listed above) in 400 ml plastic containers under the incubation conditions described above. Solidified (7 g l^{-1} agar) microtuberization media was used since it favoured the tuberization of nodal cuttings in preliminary experiments.

Microtuber production using layered shoots vs nodal cuttings

A factorial experiment, consisting of 18 treatment combinations of cultivars, growth regulators and culture types (layered shoots and nodal cuttings) was conducted using a randomized complete block design (RCBD). The experiment was repeated 3 times. Thirty-six shoots, or 216 nodal cuttings per treatment combination were harvested after 28 and 56 days of incubation.

Effect of growth regulators and duration of incubation on the microtuberization of layered shoots

A factorial experiment, consisting of 18 treatment combinations of cultivars (Kennebec, Russet Burbank and Superior), growth regulators (coumarin, CCC-BA and control medium) and duration of incubation periods (28 and 56 days) was conducted using a RCBD. The experiment was repeated 7 times. Seventy-two shoots per treatment combination were used in this experiment.

Data analysis

Microtuber weights and numbers were compared on a per original shoot basis (per layered shoot or per six nodal cuttings). The data was analyzed using the Analysis of Variance (ANOVA) and treatment means were separated by the Student-Newman-Keuls' Test (Steel & Torie 1980). The homogeneity of microtubers was evaluated by analyzing the variance of individual microtuber weights using Log-ANOVA. Microtuber fresh weight distributions were analyzed using Chi-square test of independence (Scherrer 1984).

Results

Microtuber production using layered shoots vs nodal cuttings

Microtuber weight per shoot was significantly influence by cultivar, culture type, growth regulator and by both cultivar-culture type and cultivar-growth regulator interactions (Table 1). After 56 days, nodal cuttings produced significantly lower microtuber weights than layered shoots when compared on a per shoot basis (6 nodes), but the combined number of microtubers was significantly greater using nodal cuttings than for layered shoots (Table 1).

Layered shoots of 'Russet Burbank' and 'Superior' produced significantly greater microtuber weight and number per shoot than 'Kennebec'. The addition of CCC-BA to the microtuberization medium significantly reduced microtuber weight per shoot for 'Kennebec' and 'Superior' compared with the control medium. Microtuber number per shoot was significantly reduced for 'Kennebec' when CCC-BA and for 'Russet Burbank' when coumarin and CCC-BA were respectively added to the microtuberization medium compared with the control.

Nodal cuttings of 'Russet Burbank' produced a greater microtuber weight per shoot than 'Kennebec' and 'Superior', while the latter two had similar microtuber weight per shoot. For all cultivars, nodal cuttings grown on medium containing CCC-BA produced a lower microtuber weight per shoot compared with the control medium, which produced a lower microtuber weight per shoot compared with the coumarin medium (Table 1). The duration of the incubation period did not affect the numbers of microtubers per shoot in either layered shoots or nodal cuttings. However, microtuber fresh weight per shoot was significantly greater after 56 days of incubation compared with 28 days (data not shown).

Effect of growth regulators and duration of the incubation period on the microtuberization of layered shoots

Microtuber weight per shoot was significantly influenced by cultivar, growth regulator, duration of the incubation period, and by both the cultivar-growth regulator and cultivar-incubation period interactions (Table 2). After 28 days of induction, 'Superior' had a heavier microtuber weight per shoot than the other 2 cultivars (Table 2). Differences in microtuber weights between 'Superior' and 'Russet Burbank' shoots were no longer significant after 56 days, but both were significantly heavier compared with 'Kennebec'. Medium containing CCC-BA depressed microtuber weight per 'Kennebec' shoot compared with the other two media for both incubation periods. Growth regulators did not influence the microtuber weight of 'Russet Burbank' after 28 days, but the medium containing CCC-BA produced a significantly lower microtuber weight per shoot than the two other media after 56 days. The control medium produced a heavier microtuber weight per shoot after 28 days for 'Superior' than the media containing coumarin and CCC-BA. The differences between the control and coumarin-containing media were no longer significant after 56 days, but both produced heavier microtubers compared with the CCC-BA medium. CCC in combination with BA promoted microtubers more effectively than either used alone, while coumarin was more effective used alone (data not shown).

Microtuber number per shoot was influenced by the cultivar and growth regulator treatments while the variance of microtuber weight was influenced by the duration of the incubation period (Table 2). Microtuber number per shoot was significantly less for 'Kennebec' than for the other 2 cultivars after 28 and 56 days of incubation. Growth regulators did not influence the number of microtubers produced per shoot, except for 'Kennebec' where the medium containing CCC-BA induced fewer microtubers per shoot than the other two media. After 56 days of incubation, the CCC-BA medium again induced fewer microtubers per shoot in 'Kennebec' and in 'Russet Burbank', compared with the control medium.

The Chi-square analysis of the frequency distribution of microtuber fresh weight classified according to 4 categories (≤ 0.25, 0.25–0.50, 0.50–1.0 and > 1.0 g) indicated a significant effect of culture type and length of the incubation period treatments on the frequency distribution of microtuber weight (Table 3). Microtubers produced using layered shoots were heavier than those produced using nodal cuttings. Up to 40% of the microtubers produced using the layered shoot technique had a individual weight greater than 500 mg. For layered shoots, increasing the incubation period from 28 to 56 days reduced the frequency of smaller microtubers (≤ 250 mg) and increased the frequency of microtubers > 1.0 g. Growth regulator treatments influenced the distribution of microtuber fresh weights for all three genotypes. Coumarin increased individual microtuber weight as indicated by the frequency distribution analysis. Coumarin increased the number

Table 1. Microtuber fresh weight and number per original shoot as affected by cultivar, growth regulator and culture type after 56 days of incubation.[1]

Treatments	Microtuber weight (mg) per shoot		Microtuber number per shoot	
	Layered shoot	Nodal cuttings	Layered shoot	Nodal cuttings
Kennebec				
Coumarin	2146 a[2]	655 a	3.2 a	6.1 a
CCC-BA	1622 b	204 c	2.5 b	5.7 a
Control	1981 a	452 b	3.3 a	6.0 a
Mean	1916 y(b)[3]	446 z(b)	3.0 z(b)	5.9 y(a)
Russet Burbank				
Coumarin	2276 a	840 a	3.5 b	5.9 a
CCC-BA	1801 a	264 c	3.6 b	5.5 a
Control	2202 a	664 b	4.6 a	6.2 a
Mean	2093 y(a)	629 z(a)	3.9 z(a)	5.8 y(a)
Superior				
Coumarin	2093 ab	424 a	4.0 a	6.0 a
CCC-BA	1795 b	194 c	3.6 a	6.0 a
Control	2304 a	366 b	3.8 a	5.9 a
Mean	2064 y(a)	358 z(b)	3.8 z(a)	6.0 y(a)
Significance				
Source of variation	F value	Prob	F value	Prob
Cultivar (C)	14.7	0.0001	10.5	0.0001
Culture type (T)	1276.5	0.0001	142.2	0.0001
C × T	12.2	0.0001	3.5	0.032
Growth regulator (G)	44.4	0.0001	1.6	0.19
C × G	4.0	0.004	1.1	0.38
G × T	2.7	0.07	6.8	0.0016
C × G × T	2.7	0.06	0.9	0.44

[1] The experiment was repeated 3 times. Each observation represents the average of 36 shoots or 216 nodal cuttings.
[2] Means within cultivar-incubation time combination followed by the same letter are not significantly different at the 0.05 level (Student-Neuman-Keuls' Test, SNK).
[3] Incubation period means within each cultivar followed by the same letter (y or z) are not significantly different at the 0.05 level (SNK). Cultivar means within each culture type followed by the same letter (in parenthesis) are not significantly different at the 0.05 level (SNK).

of larger (> 1 g) microtubers compared with CCC-BA and, to a lesser extent, with the control medium. For nodal cuttings, increasing the incubation period from 28 to 56 days had a limited effect on fresh weight distribution; nearly all microtubers weighed less than 250 mg. Growth regulator treatments had no influence on microtuber frequency distribution except for 'Russet Burbank' after 56 days.

The comparison of the variances of microtuber fresh weights, when produced from layered shoots and nodal cuttings after 28 and 56 days, showed that the variance increased with incubation period for both

Table 2. Microtuber fresh weight and number per original shoot as affected by cultivar, growth regulator and duration of the incubation period.[1]

Treatments	Microtuber weight (mg) per shoot		Microtuber number per shoot	
	28 days	56 days	28 days	56 days
Kennebec				
Coumarin	1178 a[2]	2102 a	3.3 a	3.4 a
CCC-BA	792 b	1314 b	2.6 b	2.8 b
Control	1042 a	1992 a	3.4 a	3.4 a
Mean	1004 z(c)[3]	1802 y(b)	3.1 y(b)	3.2 y(b)
Russet Burbank				
Coumarin	1164 a	2292 a	3.6 a	3.9 b
CCC-BA	945 a	1838 b	3.8 a	3.5 b
Control	1026 a	2137 a	4.6 a	4.9 a
Mean	1045 z(b)	2085 y(a)	4.0 y(a)	4.0 y(a)
Superior				
Coumarin	1501 b	2044 ab	4.2 a	4.0 a
CCC-BA	1253 b	1842 b	3.8 a	3.8 a
Control	1624 a	2351 a	4.2 a	4.0 a
Mean	1459 z(a)	2174 y(a)	4.1 y(a)	3.9 y(a)
Significance				
Source of variation	F value	Prob	F value	Prob
Cultivar (C)	25.6	0.001	14.6	0.0001
Growth regulator (G)	31.3	0.001	6.7	0.001
C × G	4.1	0.003	1.5	0.20
Incubation period (I)	239.7	0.0001	3.2	0.06
C × I	6.3	0.002	0.5	0.58
G × I	2.7	0.07	0.0	0.99
C × G × I	1.3	0.24	1.5	0.20

[1] The experiment was repeated 7 times. Each observation represents the average of 72 shoots.
[2] Means within cultivar-incubation time combination followed by the same letter are not significantly different at the 0.05 level (Student-Neuman-Keuls' Test, SNK).
[3] Incubation period means within each cultivars followed by the same letter (x or y) are not significantly different at the 0.05 level (SNK). Cultivars means within each incubation period followed by the same letter (in parenthesis) are not significantly different at the 0.05 level (SNK).

types of culture and that it was greater for layered shoots than for nodal cuttings (Table 4).

Discussion

The microtuberization system reported here permitted the rapid and extensive tuberization (up to 2 g of microtuber fresh weight per original shoot) of in vitro shoots of 'Kennebec', 'Russet Burbank' and 'Superior' in an

Table 3. Effect of the duration of incubation, cultivar, growth regulator and culture type on the frequency distribution (%) of microtubers.[1]

Treatments	Microtuber size category (g)					
	Layered shoot				Nodal cuttings	
	≤ 0.25	0.25-0.50	0.50-1.0	> 1.0	≤ 0.25	0.25-0.50
28 days[2]						
Kennebec						
Coumarin	64	20	10	6	100	0
CCC-BAP	59	26	14	1	100	0
Control	70	13	12	5	100	0
Russet Burbank						
Coumarin	54	22	19	5	100	0
CCC-BAP	65	23	11	1	100	0
Control	68	20	10	2	100	0
Superior						
Coumarin	66	15	13	6	100	0
CCC-BAP	66	17	15	2	100	0
Control	61	19	14	6	100	0
56 days						
Kennebec						
Coumarin	35	17	25	23	96	4
CCC-BAP	40	23	29	8	99	1
Control	34	27	20	19	98	2
Russet Burbank						
Coumarin	31	24	23	22	88	12
CCC-BAP	32	28	21	19	98	2
Control	50	18	20	12	99	1
Superior						
Coumarin	34	36	20	10	99	1
CCC-BAP	37	20	26	17	98	2
Control	39	17	23	21	99	1

[1] The experiment was repeated 3 times. The tubers of 36 shoots per treatment combination were used in this analysis.
[2] Significant X^2 values: Period $p \leq 0.001$; Explant type $p \leq 0.001$; Russet Burbank 30 days: Coumarin (COU) vs CCC-BAP (CCC) $p \leq 0.006$, COU vs Control (CON) $p \leq 0.011$, 60 days: COU vs CON $p \leq 0.011$, Nodal cutting: COU vs CCC $p \leq 0.016$, COU vs CON $p \leq 0.001$; Kennebec 30 days: COU vs CCC $p \leq 0.026$, CCC vs CON $p \leq 0.008$, 60 days: COU vs CCC $p \leq 0.026$; Superior 30 days: COU vs CCC $p \leq 0.02$, CON vs CCC $P \leq 0.008$, 60 days: COU vs CCC $p \leq 0.040$, COU vs CON $p \leq 0.017$.

MS based medium containing elevated sucrose level alone and incubated under an 8 h photoperiod at 15 °C.

Microtuber production using layered shoots vs nodal cuttings

When layered shoots and nodal cuttings were incubated for the same length of time, individual microtuber fresh weight and fresh weight per original shoot produced from layered shoots was much greater than for nodal cuttings. After 56 days under an inductive environment the microtuber weight from layered shoots was 3–5 times greater than for nodal cuttings. Increased microtuber fresh weight from layered shoots compared with nodal cuttings may be due to increased production of endogenous growth regulators and gradients of these growth regulators. In addition, the increased leaf surface area available for the perception of the

Table 4. Effect of the duration of incubation period and culture type on the variance of microtuber fresh weight.[1]

Incubation period (days)	Microtuber fresh weight variance (10^3 mg)	
	Layered shoot	Nodal cuttings
28	52.3 b[2]	0.9 d
56	161.8 a	1.8 c

[1] The tubers of 324 shoots per treatment were used in this analysis.
[2] Means followed by the same letter are not significantly different at the 0.05 level (Student-Neuman-Keuls' Test).

photoperiodic stimuli and larger surface area in contact with the medium may have contributed to the increased microtuber production. Favouring vegetative growth of stolon segments and shoots prior to microtuber induction have been reported to increase microtuber fresh weight (Chapman 1955; Garner & Blake 1989). Clearly, greater microtuber yield can be achieved by increased vegetative area of cultures prior to microtuber induction.

Although layered shoots produced heavier microtuber weight per original shoot, nodal cuttings produced more microtubers. This difference in microtuber number per original shoot is most likely due to the expression of correlative inhibition in layered shoots even though correlative inhibition may be somewhat altered by changing the orientation by 90 °C. When shoots are dissected into nodal cuttings their hormonal balance is disrupted, promoting microtuberization of each nodal cutting.

Effect of growth regulators and duration of the incubation period on the microtuberization of layered shoots

The addition of coumarin, CCC and BA to the microtuberization medium of layered shoots failed to trigger any increase in fresh weight or number of microtubers per shoot compared with elevated sucrose alone and may have depressed microtuber fresh weight per shoot to some extent (Table 2). These results contradict previous reports of the beneficial effect of these compounds on the microtuberization of isolated stolons, stem segments or nodal cuttings (Palmer & Smith 1969, 1970; Forsline & Langille 1976; Stallknecht & Farnsworth 1982a, 1982b; Hussey & Stacey 1984; Pelacho & Mingo-Castel 1991). The promotive effect of cytokinins and CCC, used singly, on microtuberization has previously been questioned (Stallknecht 1985; Ewing 1990). The discrepancies observed in the effect of growth regulators on microtuberization are likely related to the nature and specific endogenous hormonal content of the plant material used to induce microtubers. Under a short photoperiod (8 h) and cool temperature (15 °C), the addition of coumarin, CCC or BA to the microtuberization medium of layered shoots is unnecessary and may even be deleterious.

The advantage of the technique outlined here over Garner & Blake's (1989) is that heavier microtubers can be produced more rapidly. One potential drawback of this method is the increased heterogeneity in microtuber weight.

In conclusion, microtuberization of shoots can be achieved rapidly by layering them in a modified MS based liquid propagation medium containing 20 g l^{-1} sucrose under 16-h light period at 22 °C for 28 days, and then replacing the residual medium by an MS based liquid microtuberization medium containing 80 g l^{-1} sucrose under 8-h light period at 15 °C. With this method, exogenously supplied coumarin, CCC and BA are not necessary for optimal microtuber production. This microtuberization system, a simplified version of the protocol developed by Estrada et al. (1986) and Meulemans et al. (1986), may have significant commercial implications. The use of liquid media, the absence of growth regulators in the microtuberization media and the lower labour input required to handle shoots could reduce the production cost of microtubers. The production of heavier microtubers could substantially increase yield of these propagules in the greenhouse or in the field (Wiersema et al. 1987). Additional efforts are required to further increase the productivity of this system. The application of this microtuberization system to the study of potato tuberization may prove valuable, since both aspects of tuber formation, i.e. the perception of environmental stimulation and the reaction to this stimulation, can be studied simultaneously in vitro.

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