Growth Estimation of *in vitro* Cultured Callus and Plant Regeneration from Leaf Disk and Petiole Callus of Musk Strawberry (*Fragaria moschata* Duch.) Subcultured for 18 Months

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Summary

Callus was induced from leaves and petioles of *in vitro* cultured *Fragaria moschata* shoots. The highest callus formation rate was obtained on MS supplemented with NAA $1.0 \text{ mg} \cdot \text{l}^{-1}$ and BA $0.025 \text{ mg} \cdot \text{l}^{-1}$. These calli were transferred to eight MS media supplemented with different combinations of NAA and BA. After 18 months with periodic subcultures, callus growth was estimated by their area during a 8-week culture period. The highest growth rate was observed on medium supplemented with NAA $0.1 \text{ mg} \cdot \text{l}^{-1}$ and BA $0.25 \text{ mg} \cdot \text{l}^{-1}$ and the lowest on the one supplemented with NAA $0.5 \text{ mg} \cdot \text{l}^{-1}$ and BA $0.025 \text{ mg} \cdot \text{l}^{-1}$. Callus weight was measured to determine the fidelity of the callus area calculation method as a non-destructive way of estimation the growth rate. High correlation was observed between callus area and callus weight ($r^2=0.974$ to 0.996). After 18 months in culture, calli were transferred to different regeneration-inducing media enriched with TDZ, BA, ZEA, IBA and NAA. After nearby four months on these media, the calli regenerated shoots which when transferred to a root-inducing medium, they formed normal roots.

Key Words: *Fragaria moschata*, callus growth, regeneration.

Introduciton

Callus culture can be obtained from any organ of plant (Yeoman and MacLeod, 1977). *in vitro* callus formation is a phenomenon similar to proliferation of tissues after wounding an *in vitro* growing plant, *in vitro* callus induction usually needs an auxin in the medium (Yeoman and MacLeod, 1977). Once induced, active cell proliferation can be maintained at high rates if the callus is periodically transferred to fresh medium (Dixon, 1985).

Plant regeneration from strawberry calus was reported for the first time by Jones *et al.* in 1988. One purpose of regenerating plants from callus is to seek a useful somaclonal variant. In spite of the endeavours conducted during recent years, there is only one example of a *Fragaria* somaclonal regenerant with trait superior to the original clone: *F. × ananassa* 'Hokowase' resistant to *Fusarium* wilt (Toyoda *et al.*, 1991).

The reduciton and even the total suppression of the regeneration ability are common phenomena observed during undifferentiated cell culture. Nehra *et al.* (1990) showed that callus culture of *F. × ananassa* 'Redcoat' after 24 weeks, complete lost its regeneration capacity. This inability was explained by the formation of cells

with abnormal DNA content during the unorganized cell growth phase.

There are no reports concerning the determination of the callus growth rate on different media and the plant regeneration of any *Fragaria* species after more than 18 months of culture.

Materials and Methods

Callus induction

Fragaria moschata cultures maintained for 4-5 weeks on Murashige and Skoog, MS (1962) medium supplemented with $1 \text{ mg} \cdot \text{l}^{-1}$ BA, $30 \text{ g} \cdot \text{l}^{-1}$ sucrose and $6.5 \text{ g} \cdot \text{l}^{-1}$ agar (Bacto-Difco), pH 5.7 were used for callus induction.

Transversally cut leaves and petioles (5 mm) were placed on 90 mm Petri dishes containing MS mineral salts and vitamins supplemented with 0.1, 0.5 and $1.0 \text{ mg} \cdot \text{l}^{-1}$ naphthalene acetic acid (NAA), 0.05 and $0.25 \text{ mg} \cdot \text{l}^{-1}$ 2,4-dichlorophenoxy acetic acid (2,4-D) combined with 0 and $0.025 \text{ mg} \cdot \text{l}^{-1}$ 6-benzylaminopurine (BA). Cultures were maintained in darkness at 23 ± 2 °C. Callus growth was measured after 3 weeks of culture using the following scale: 0 (no callus formation), 1 (callus diameter $< 2 \text{ mm}$), 2 (callus diameter 3-4 mm), 3 (callus diameter $> 5 \text{ mm}$).

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Callus culture

After the induction phase, the largest callus derived from the original tissues were transferred to Petri dishes containing MS salts and vitamins, 30 g·l⁻¹ sucrose, 2 g·l⁻¹ Phytigel (Sigma), 1.0 mg·l⁻¹ NAA and 0.025 mg·l⁻¹ BA. Cultures were maintained at 23 ± 2 °C under a 16-h photoperiod, photosynthetic photon flux density (PPFD) 35-40 μmol·m⁻²·s⁻¹. The callus was subcultured 3 times at a 4-week interval. Afterwards, calli were transferred to eight different media based on the MS mineral salts and vitamins, 30 g·l⁻¹ sucrose, 6.5 g·l⁻¹ agar Bacto-Difco and pH 5.7. The growth regulators used were 0.1, 0.5, 1.0 and 2.0 mg·l⁻¹. NAA combined with 0.025 and 0.25 mg·l⁻¹ BA. After 18 months of culture with subcultures made every 4-5 weeks, calli were used for the growth-curve determination.

Four calli (90 mm²) were placed on five 90 mm Petri dishes. Callus growth was determined through the calculation of their area, according to Taylor and Secor (1992). Twenty calli per each medium were evaluated for an 8-week culture period. The data were analyzed by the Student-Newman-Keuls multiple comparison test (p=0.05).

Plant regeneration

Eighteen month old calli periodically subcultured on MS mineral salts and vitamins, sucrose 30 g·l⁻¹, agar Bacto-Difco 6.5 g·l⁻¹, NAA 1 mg·l⁻¹, BA 0.025 mg·l⁻¹ (pH 5.6) were used for regeneration.

Regeneration-inducing media contained the MS or the Shenk and Hildebrandt (SH) (1972) mineral salt medium, 30 g·l⁻¹ sucrose, 6.5 g·l⁻¹ agar Bacto-Difco (pH 5.6) supplemented with 1-phenyl-3-(1, 2, 3-thiadiazol-5-yl) urea (TDZ), BA, Zeatin (ZEA), NAA, Indolbutyric acid (IBA) (Table 3). Petri dishes were maintained under a 16-h photoperiod PPFD 30 μmol·m⁻²·s⁻¹.

Calli were treated by the following two methods: a) maintenance for 120 days on media described on Table 3, with a subculture on the same media after the first 90 days, or b) induction for 7 or 14 days and definitive transfer to MS 0 (MS medium without growth regulator) or transfer for 30 days to MS 0 and then to MS 1 mg·l⁻¹ TDZ, 0.01 mg·l⁻¹ NAA or SH, 1 mg·l⁻¹ TDZ, 0.01 mg·l⁻¹ IBA (Table 4).

Regeneration frequency is expressed as the % of callus with a differentiated shoot. Regenerants were transferred to a root-inducing medium: B 5 minerals salts (Gamborg *et al.*, 1968) and MS vitamins, sucrose 30 g·l⁻¹, agar Bacto-Difco 6.5 g·l⁻¹, IBA 1 mg·l⁻¹, (pH 5.6).

Results and Discussion

Callus induction

Calli developed mainly on ends of leaf veins and cut

surfaces of petioles. Medium enriched with 0.1 mg·l⁻¹ NAA and 0.025 mg·l⁻¹ BA induced callus formation on 90% of small explants (callus size=1). No difference on callus induction frequency was observed between the two organs. NAA at the highest concentration used produced the best results. Media supplemented with 2, 4-D induced 30-50% of the organs to form callus (Table 1).

Callus culture

Our data confirmed that the callus area is usable to estimate callus growth rate without disturbing cultures. Correlation between fresh weight (g) and callus area (mm²), regardless of the growth regulator combination used, resulted in r² value between 0.974 and 0.996 (Table 2).

Medium supplemented with NAA 0.1 mg·l⁻¹ and BA 0.25 mg·l⁻¹ induced the highest growth rate, of nearly 7 fold in callus area after 8 weeks of culture. On media with 0.025 mg·l⁻¹ BA, callus growth increased proportionally to NAA concentration. On the contrary, on media supplemented with 0.25 mg·l⁻¹ BA, the highest

Table 1. Callus formation frequency (%) on transversally cut leaves and petioles of *Fragaria moschata* after three weeks in culture on MS medium supplemented with different growth regulator combinations.

Growth regulators (mg·l ⁻¹)			Callus formation (%)		Callus size (*)	
2, 4-D	NAA	BA	Leaf	Petiole	Leaf	Petiole
0.05	—	—	—	—	0	0
0.05	—	0.025	—	—	0	0
0.25	—	—	30	35	1	1
0.25	—	0.025	50	45	1	1
—	0.1	—	80	80	2	2
—	0.1	0.025	90	90	1	1
—	0.5	—	60	70	2	2
—	0.5	0.025	45	65	2	2
—	1.0	—	80	90	3	2
—	1.0	0.025	100	90	3	2

(*) 0 = no callus formation; 1 = callus diameter < 2 mm; 2 = callus diameter 3-4 mm and 3 = callus diameter > 5 mm

Table 2. Correlation between callus area (A) in square millimeters and weight (W) in grams of *Fragaria moschata* cultures on MS medium supplemented with different growth regulator combinations.

Growth regulators (mg·l ⁻¹)		Correlation curve	r ²
NAA	BA		
0.1	0.025	W = -0.100 + 0.004·A	0.980
0.5	0.025	W = -0.100 + 0.005·A	0.980
1.0	0.025	W = -0.207 + 0.005·A	0.974
2.0	0.025	W = -0.272 + 0.060·A	0.994
0.1	0.25	W = -0.125 + 0.004·A	0.990
0.5	0.25	W = -0.075 + 0.005·A	0.990
1.0	0.25	W = -0.260 + 0.060·A	0.982
2.0	0.25	W = -0.292 + 0.006·A	0.996

growth was obtained with the lowest NAA concentration. Comparing growth curves at both levels of BA at the same NAA concentration, the highest growth with $0.25 \text{ mg} \cdot \text{l}^{-1}$ BA was registered only with the lowest NAA concentration. The contrary was observed at 1 and $2 \text{ mg} \cdot \text{l}^{-1}$ NAA; at near $1 \text{ mg} \cdot \text{l}^{-1}$ NAA, there is no

difference in growth at both BA concentrations tested (Fig. 1).

The $0.025 \text{ mg} \cdot \text{l}^{-1}$ BA curve could be defined in the majority of the zones as linear, and only in some zones, quadratic. The $0.25 \text{ mg} \cdot \text{l}^{-1}$ BA curve, on the contrary is mainly cubic, in some zones quadratic, but never linear.

Fig. 1. *Fragaria moschata* callus area evolution during an 8-week period on MS media enriched with $0.025 \text{ mg} \cdot \text{l}^{-1}$ BA (●) and $0.25 \text{ mg} \cdot \text{l}^{-1}$ BA (★) and combined with four levels of NAA. (a) one week; (b) two weeks; (c) three weeks; (d) four weeks; (e) five weeks; (f) six weeks; (g) seven weeks and (h) eight weeks.

This response suggests a different growth regulator interaction between NAA and BA concentrations.

Plant regeneration

After 40 days in culture, no organogenesis was observed. Calli looked dark brown, especially on those induced on media enriched with high growth regulator concentrations (Table 4). After 80 days in culture, a meristematic region of light green color over the dark surfaced formed; calli maintained on MS media (Table 3) also appeared the same, whereas calli maintained on SH media did not develop any light green zones.

The highest regeneration frequency (80%) was obtained with calli induced for 7 days on a SH supplemented with $5 \text{ mg} \cdot \text{l}^{-1}$ TDZ, and $0.1 \text{ mg} \cdot \text{l}^{-1}$ NAA, and then transferred to MS 0 for 30 days and returned to SH medium with $1 \text{ mg} \cdot \text{l}^{-1}$ TDZ, $0.01 \text{ mg} \cdot \text{l}^{-1}$ IBA. On those calli induced on the MS media transferred to MS 0 for 30 days and then to MS containing $1 \text{ mg} \cdot \text{l}^{-1}$ TDZ and $0.01 \text{ mg} \cdot \text{l}^{-1}$ NAA, 50-70% of the calli regenerated shoots (Table 4).

Low regeneration frequency was registered on media described on Table 3. On the other hand, calli induced on a media with high growth regulator concentration

media and then transferred to MS 0 for all the culture period, did not regenerate any shoot or root.

When regenerants were transferred to the root-inducing medium, all formed normal roots; some formed embryo-like structures on leaf edges.

Conclusions

The callus induction response of this genotype to the growth regulators used seems different from the one observed with other *Fragaria* species: 2, 4-D combined with BA induced the highest callus formation on *F. vesca* (Bois, 1992), whereas on *F. moschata*, 2, 4-D induced the formation of little compact callus which did not grow. The callus formed with NAA was white and friable, and continued to grow for long periods.

Subculturing of *Fragaria* callus has been reported to reduce the regeneration capacity because of continued divisions of undifferentiated cells (Nehra *et al.*, 1990). This inability could be associated by genetic disorders which occur during rapid cell division, such as the variation on the DNA content producing polyploids, endoreplication, and deletion that preclude normal plant regeneration (Nehra *et al.*, 1991; Castorena Sanchez *et al.*, 1988). Infante *et al.*, (1994) observed that on *Fragaria* species with higher ploidy level, the formation of cells with abnormal DNA content is much more frequent than it is in diploids. It seems that the regeneration phenomena requires a normal complement of DNA in cells.

This is the first report that describes a stable *Fragaria* callus culture, and the only example of this genera in which the regeneration capacity was maintained after 18 months of subculturing.

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Table 3. Regeneration frequency (%) of *Fragaria moschata* from callus after 120 days on different MS (1962) and SH (1972) media.

Media	Growth regulators ($\text{mg} \cdot \text{l}^{-1}$)				(%)
	TDZ	BA	ZEA	IBA	
MS	0.5	—	—	0.01	25
MS	1.0	—	—	0.01	25
MS	—	—	1.0	0.05	0
MS	—	1.0	0.1	0.10	0
SH	0.5	—	—	0.01	0
SH	1.0	—	—	0.01	0
SH	—	—	1.0	0.05	0
SH	—	1.0	0.1	0.10	0

Table 4. Regeneration frequency (%) of *Fragaria moschata* from callus after induction on different MS (1962) and SH (1972) media.

Media	Growth regulators ($\text{mg} \cdot \text{l}^{-1}$)		Treatment	(%)
	TDZ	NAA		
MS	0.5	—	7 days + X	0
MS	1.0	—	14 days + X	0
SH	—	—	7 days + X	0
SH	—	—	14 days + X	0
MS	0.5	0.1	7 days + Y	70
MS	1.0	0.1	14 days + Y	50
SH	—	0.1	7 days + Z	80
SH	—	0.1	14 days + Z	0

(*) ^x definitive transfer to MS 0

^y 30 days on MS 0 and transfer to MS $1 \text{ mg} \cdot \text{l}^{-1}$ TDZ, $0.01 \text{ mg} \cdot \text{l}^{-1}$ NAA.

^z 30 days on MS 0 and transfer to SH $1 \text{ mg} \cdot \text{l}^{-1}$ TDZ, $0.01 \text{ mg} \cdot \text{l}^{-1}$ IBA.

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シロバナヘビイチゴ (*Fragaria moschata* Duch.) ‘カプロン’の試験管培養におけるカルス生長と個体再生

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摘 要

試験管内で培養したシロバナヘビイチゴ (*Fragaria moschata*) のシュートから葉および葉柄を外植体として、MS 培地上でカルスを誘導した。培地に NAA 1 mg・l⁻¹ と BA 0.025 mg・l⁻¹ を添加した場合、カルス形成速度はもっとも高かった。その後、NAA と BA の異なる組合せの MS 培地に移植、18 ヶ月間継代培養したカルスを供試して、各培地での生長量を比較した。生長量はシャーレ上で8週間培養した時の、カルス増加面積として表した。最も良好な生長は、NAA 0.01 mg・l⁻¹ と BA 0.25 mg・l⁻¹ の組合せで観察され、

NAA 0.5 mg・l⁻¹ と BA 0.025 mg・l⁻¹ の組合せで最も劣った。面積で求めた生長量が実際のカルス生長に見合うか、カルス生体重を計測したところ、高い相関が得られた ($r^2=0.974 \sim 0.996$)。

18 ヶ月間培養されたカルスの個体再生の可能性をみるために、TDZ, BA, ZEA, IBA, NAA を含む培地に移植した。その結果、4 ヶ月後にシュート形成がみられ、このシュートを発根培地へ移植したところ、健全な発根が認められた。