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Recovering vitrified carnation (*Dianthus caryophyllus* L.) shoots using Bacto-Peptone and its subfractions

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Abstract. Vitrified shoots regenerated from carnation petals (*Dianthus caryophyllus* L. cv. Scania) were recovered by culturing them in a medium containing 3.0 g/l Bacto-Peptone. Wax structures not found on vitrified shoots developed on the abaxial surface of leaves of recovered shoots and on those of normal leaves. Recovered shoots were rooted and successfully acclimatized while vitrified shoots could not survive the acclimatization process. The Bacto-Peptone solution was fractionated and the efficiency of each fraction for the recovery of vitrification was examined. Only basic, non high molecular fractions whose molecular weight was less than 10,000 were effective.

Key Words: *Dianthus caryophyllus* L., Vitrification, Plant Tissue Culture

Introduction

Vitrification is a knotty problem which often occurs in in-vitro plantlets regenerated from cultured cell or tissue (Kevers et al 1984). Vitrified shoots are abnormally glassy, thick and translucent. In almost all cases, it is impossible to obtain normal mature plants from them. There are quite a few reports attempting to solve the problem of vitrification. The types and concentration of carbohydrates in the medium (Rugini 1986, Orlikowska 1987), light intensity (Sutter et al. 1979), photoperiod (Gimelli et al. 1984), and the types and concentration of gelling agents in the medium (Thomas et al. 1989, Leshem 1983ab, Hakkaart et al. 1983) are reported to affect the frequency of vitrified shoots. However, all of these treatments have no effect on the recovery of vitrified shoots. So far, there is no report which has succeeded in obtaining normal plants from vitrified shoots. In the present work, normal plantlets were obtained from vitrified shoots by transferring them to a medium containing Bacto-Peptone.

Materials and Methods

Plant material Carnation (*Dianthus caryophyllus* L. cv. Scania) plants were grown in a greenhouse.

Culture of petals Petals were excised from immature flower buds (10–15 mm diameter) before anthesis and cultured in test tubes (3 cm diameter, 10 cm height) covered with two-fold aluminium foil containing 10 ml of Murashige and Skoog's medium (1962) supplemented with 30 g/l sucrose, 0.2% Gelrite (Wako Pure Chemical Industries Ltd., Osaka, Japan), 0.5 mg/l 1-naphthaleneacetic acid (NAA), and 0.5 mg/l benzylaminopurine (BAP) at 25°C under a 16 h light (white fluorescent lamp, 7.25 w/m^2)– 8 h dark cycle.

Recovery of vitrified shoots Vitrified shoots regenerated from the petals were transferred to a medium containing 1.0–5.0 g/l Bacto-Peptone (Difco, Michigan, USA), 3.0 g/l Hyponex 6.5-6-19 (Hyponex Corporation, Ohio, USA), 30 g/l sucrose

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and 0.8% agar (recovery medium) and cultured under the same conditions. This medium was a modified Kakehi (1979) medium used for rooting of cultured shoots of carnation. After 5 weeks, these shoots were transferred to Murashige and Skoog's medium containing 30 g/l sucrose and 0.2% Gelrite but no plant growth substances in order to induce rooting. The recovery effects of four types of Poly Peptone (Japan Pharmaceutical Ltd., Tokyo), that is, Poly-Peptone (an enzymatically hydrolyzed milk casein), Poly-Peptone-P1 (an enzymatically hydrolyzed animal meat), Poly-Peptone-S (an enzymatically hydrolyzed defatted soybean), Poly-Peptone-Y (an enzymatically hydrolyzed yolk protein), and casamino acids (Difco, Michigan, USA) on the vitrified shoots were also examined by adding 3.0 g/l to the recovery medium in place of Bacto-Peptone. Vitrified shoots were transferred to media containing one of these substances, cultured for 5 weeks, then observed.

Acclimatization Rooted shoots in-vitro were potted using Perlite (Nenisanso No. 1, Mitsui Mining and Smelting Company Ltd., Tokyo) as the substrate and grown in a mistroom setting in a greenhouse. The temperature in the greenhouse was maintained between 15℃ and 30℃. In the mistroom, relative humidity (RH) was controlled by a humidifier and light intensity was reduced by shading. During the first week, the mistroom was maintained at 100% RH and 80% shaded. During the second week, the mistroom was maintained at 80% RH and 80% shaded. In the third week, humidification in the mistroom was stopped but 80% shading was maintained. On the first day of the fourth week, the plants were moved out of the mistroom, grown for 1 week, then transferred to soil in pots and grown in the greenhouse.

Fractionation of Bacto-Peptone 1) Separation by molecular weight at M.W. 10,000 was performed by ultrafiltration using Centricon-10 (W. R. Grace Co. MA, USA). The Centricon-10 was a cylindrical form 16 mm in diameter, 126 mm in length, which was composed of an ultrafiltrate membrane, membrane support base, sample reservoir, filtrate cap and retentate cap. A 2.5 ml of Bacto-Peptone solution (0.15 g/ml distilled water) was put into the Centricon-10 and centrifuged at 5,000 g. for 8 h. Transudate in the filtrate cap was collected. Distilled water (2 ml) was put into a unit of Centricon-10 and centrifuged again at 5,000 g. for 2 h. This was repeated 4 times. Transudates in the filtrate cap were collected and put together. The solution was now composed of substances lower than 10,000 in molecular weight. The remainder on the membrane was collected as follows. The centricon-10 was inverted and centrifuged at 1,000 g. for 10 min. High molecular fractions composed of substances higher than 10,000 in molecular weight were collected in the retentate cap. Ten ml of Bacto-Peptone solution (0.15 g/ml) was fractionated. 2) Bacto-Peptone solution was separated into acidic, basic and neutral fractions by ion exchange chromatography using Dowex 50W-X4 and Dowex 1W-X2 (The Dow Chemical Company, USA). A solution of 1.58 g Bacto-Peptone in 10 ml distilled water was put onto a column (25 mm in diameter, 500 mm in length, 700 ml bed volume) packed with Dowex 50W-X4. The basic fraction absorbed by Dowex 50W-X4 (H^+) were eluted by 2N NH_4OH. The fraction which passed through the Dowex 50W-X4 column was put onto a column (25 mm in diameter, 300 mm in length, 350 ml bed volume) packed with Dowex 1W-X2 (OH^-). The acidic fraction absorbed by Dowex 1W-X2 was eluted by 1N HCl. The neutral fraction was the fraction which passed through the Dowex 1W-X2 column. Each fraction was concentrated by rotary vacuum evaporator at 40℃. From 1.58 g Bacto-Peptone, 0.17 g acidic fraction, 1.26 g basic fraction and 0.03 g neutral fraction were obtained.

Each of these fractions obtained from 1.5 g or 1.58 g Bacto-Peptone was added to 500 ml recovery medium in place of Bacto-Peptone to examine the recovery effect on vitrified shoots. This concentration was equivalent to that contained in 3.0 g/l Bacto-Peptone. Observation was carried out after 5 weeks of culture.

Scanning electron microscope Five millimeter square leaf segments of normal plants, vitrified shoots and recovered shoots were fixed in 0.2% osmic acid for 24 hr. Thereafter, they were coated with gold by an ion sputter (JFC-1100, JEOL,Tokyo) and observed using a scanning electron microscope(JSM T220A, JEOL, Tokyo).

Results and Discussion

Recovery of vitrification

Shoots were regenerated from cultured immature petals within 8 weeks. All of these shoots were vitrified, that is, they were translucent and not glaucous. They were rooted in a Murashige and Skoog's medium without plant growth substances (rooting medium). Vitrified plantlets obtained were transferred to an acclimatization process. However, all of them died out during this process. Vitrified shoots were recovered by transferring them to a medium supplemented with

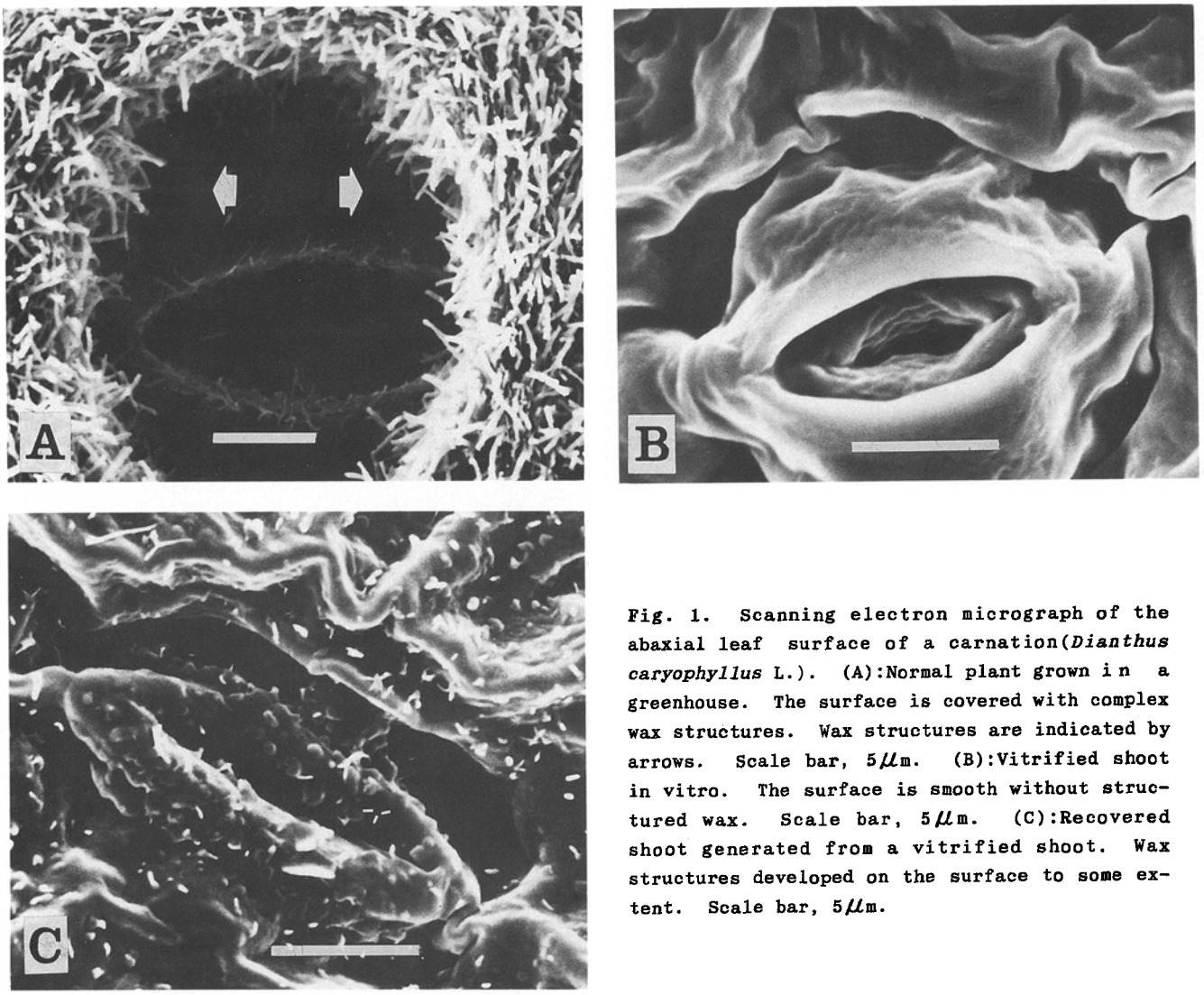

Fig. 1. Scanning electron micrograph of the abaxial leaf surface of a carnation(*Dianthus caryophyllus* L.). (A):Normal plant grown in a greenhouse. The surface is covered with complex wax structures. Wax structures are indicated by arrows. Scale bar, 5μm. (B):Vitrified shoot in vitro. The surface is smooth without structured wax. Scale bar, 5μm. (C):Recovered shoot generated from a vitrified shoot. Wax structures developed on the surface to some extent. Scale bar, 5μm.

Bacto-Peptone (Table 1). Newly generated leaves from vitrified shoots were glaucous and not translucent. The maximum recovery rate was attained with 3.0 g/l (Table 1), while 5.0 g/l inhibited the growth of shoots. The recovered shoots were rooted in the rooting medium, then transferred to ex-vitro conditions and acclimatized. Ninety two percent of them survived and grew, in contrast to the zero survival rate of vitrified plantlets (Table 2).

Surface structures of normal plants, vitrified shoots and recovered shoots

There are epicuticular wax structures on the abaxial leaf surface of normal carnation plants (Fig. 1A). In contrast, vitrified shoots completely lack the structured wax (Fig. 1B). On the abaxial leaf surface of the shoots recovered from vitrification, development of the wax structures was observed (Fig. 1C). Sutter et al. (1979) also observed leaves of in vitro cultured plantlets of carnation and reported that glaucous leaves were usually covered with epicuticular wax, as those of normal plants, whereas abnormally thickened, translucent leaves lacked such wax structures. The formation of the wax structures is considered to be closely related to the recovery of vitrified shoots.

Effect of various peptone and casamino acids

Casamino acid and several types of peptone different in their material protein were examined for their effects on the recovery of vitrified shoots (Table 3). Among them, only Poly-Peptone P1, which is an enzymatically hydrolyzed animal meat showed a somewhat higher effect than Bacto-

Table 1. Effect of the Bacto-Peptone concentration in the medium on the recovery of vitrified shoots.

Peptone conc. (g/l)	0	1.0	2.0	3.0	5.0
No. of recovered shoots	0	10	14	20	16
No. of unrecovered shoots	25	15	11	5	9
Percentage of recovery (%)	0	40	56	80	64

Vitrified shoots were transferred to media containing 0-5 g/l Bacto-Peptone and observed after 5 weeks of culture.

Twenty five vitrified shoots were tested for each concentration.

Peptone. Bacto-Peptone, which is a mixture of enzymatically hydrolyzed animal meat and milk casein, showed intermediate efficiency between that of Poly Peptone P1 and Poly Peptone, which is an enzymatically hydrolyzed milk casein. Interestingly, casamino acids, which is an acid hydrolyzed casein, showed no effect. Considering the fact that Poly-Peptone, an enzymatically hydrolyzed casein, showed significant efficiency, the active substance(s) may be peptide(s), not amino acids.

Table 2. Survival rate of vitrified and recovered shoots after the acclimatization process.

	No.of tested tested plantlets	No. of plants that survived	Rate of survival (%)
Vitrified shoots	50	0	0
Recovered shoots	50	46	92.0

Vitrified and recovered shoots were transferred to the acclimatization process and observed after 4 weeks.

Efficiency of Bacto-Peptone fractions in recovery

Bacto-Peptone was separated in two fractions where the molecular weights were lower and higher than 10,000, and the efficiency of each fraction in the recovery of the vitrified shoots was examined. The low molecular fraction, where the molecular weight was lower than 10,000, was active (Table 4). Bacto-Peptone was also separated by ion exchange resin into three fractions, that is, acidic, basic, and neutral, and the efficiency of each fraction was examined. Only the basic fraction could replace Bacto-Peptone (Table 5).

Table 3. Recovery effect of several types of peptone and casamino acids.

	Types of peptone(3.0 g/l)					
	BP[a]	PP[a]	PP1[a]	PPS[a]	PPY[a]	CA[a]
No. of tested shoots	30	30	30	30	30	25
No. of recovered shoots	19	14	21	10	6	1
Recovery rate(%)	63.3	46.7	70.0	33.3	20.0	4.0

[a]BP: Bacto-Peptone, a mixture of, animal meat and milk casein,enzymaticaly hydrolyzed. PP: Poly Peptone, an enzymatically hydrolyzed milk casein. PP1: Poly Peptone P1, an enzymatically hydrolyzed animal meat. PPS: Poly Peptone S, an enzymatically hydrolyzed defatted soybean. PPY: Poly Peptone Y, an enzymatically hydrolyzed yolk protein. CA:casamino acids, acid-hydrolyzed casein.

Vitrified shoots were transferred to media containing 3.0 g/l peptone or casamino acids. Observation of the recovery of vitrified shoots was carried out after 5 weeks of culture.

Table 4. Recovery effect on vitrified shoots of fractions of Bacto-Peptone separated by molecular weight.

	Morecular weight of fraction	
	higher than 10,000	lower than 10,000
No. of tested shoots	75	75
No. of recovered shoots	14	61
Recovery rate(%)	18.7	81.3

Vitrified shoots were transferred to media containing either a high or low molecular fraction of Bacto-Peptone at concentrations equivalent to those in 3.0 g/l Bacto-Peptone. Observation was carried out after 5 weeks of culture.

Table 5. Recovery effect on vitrified shoots of acidic, basic and neutral fractions of Bacto-Peptone.

	Fractions of Bacto-Peptone			
	Bacto-Peptone	acidic	basic	neutral
No.of tested shoots	150	150	150	150
No. of recovered shoots	85	11	87	15
Recovery rate(%)	56.3	7.3	58.0	10.0

Vitrified shoots were transferred to media containing either 0.17 g/l acidic, 1.26 g/l basic, 0.03 g/l neutral fraction or 3.0 g/l Bacto-Peptone. Observation was carried out after 5 weeks of culture.

Recovery by Bacto-Peptone probably cannot be attributed to its amino acid make-up. It may be attributed, rather, to some specific basic peptide(s) smaller than M.W. 10,000. To obtain intact plants from cultured cells or tissues is the goal of most plant tissue culture experiments. There are quite a few cases in which only vitrified shoots can be obtained or most of regenerated shoots are vitrified. The technique presented in this paper could be the solution for such problems.

References

Gimelli F, Ginatta G, Venturo R, Positan S, Buiatti M (1984) Riv Ortiflorofruttic 68:107-121

Hakkaart FA, Versluijs JMA (1983) Neth J Plant Path 89:47-53

Kakehi M (1979) Bulletin of The Hiroshima Agricultural College 6:159-166

Kever C, Coumans M, Coumans-Gilles MF, Gasper T (1984) Physiol Plant 61:69-74

Leshem B (1983a) Ann Bot 42:413-415

Leshem B (1983b) Ann Bot 52:873-876

Murashige T, Skoog F (1962) Physiol Plant 15:473-497

Orlikowska T (1987) Acta Hort 212:237-244

Rugini E (1986) Almonds. In: Handbook of Plant Cell Culture, Vol 4 (DA Evans et al.) Macmillan Pub. Co, London, pp576-611

Sutter E, Langhans RW (1979) J Am Soc Hort Sci 104:493-496

Thomas WZ, Cobb BG (1989) Plant Cell Reports 8:358-360