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Short communication

Plant regeneration from grape callus stored under a combination of low temperature and silicone treatment

T. MORIGUCHI, I. KOZAKI, N. MATSUTA & S. YAMAKI[1]
Fruit Tree Research Station, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Ibaraki, 305 Japan; ([1]present address: Faculty of Agriculture Nagoya University, Chikusa Nagoya, 464 Japan)

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Abstract. Effects of the combination of low temperature and silicone treatment on the storage of grape callus (*Vitis vinifera* L. × *V. labrusca* L. cv. Kyoho; *V. vinifera* L. cv. Koshusanjaku) were examined. In 'Kyoho', the calli were stored at 10 °C successfully for up to 360 days. Embryogenic calli of 'Koshusanjaku' stored at 10 °C retained the ability of embryogenesis after 360 days of storage. However, the color of both calli became brownish. This was improved by the combination of low temperature and silicone treatment. The calli of 'Kyoho' survived by the storage under the combination of 15 °C and silicone. Embryogenic calli stored at 10 and 15 °C in combination with silicone survived for 360 days, and regenerated only after transfer onto a regeneration medium. Thus the combination of low temperature and silicone affects the longevity of the grape callus.

Introduction

Plant callus cultures are ideal materials for the analysis of various experimental systems such as induction or isolation of variants, screening of tissues resistant to stress or toxins, production of useful secondary metabolites and production of somatic hybrids. In addition, callus cultures have been used for biochemical, physiological, genetic and pathological studies. Callus cultures are usually maintained by subculture. However, serial transfer may induce polyploidy, genetic variation, loss of ability in morphogenesis and deterioration of biosynthesis of secondary metabolites [2]. As the subculture of many clones is labor-intensive and costly, prolongation of the duration of subculture of the callus by decreasing the metabolism is a useful method for solving these problems. However, little information is

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available on the storage of callus above freezing temperature. Storage of callus through drying or the use of mineral oil has been attempted in several species, such as carrot [7, 8], periwinkle and coffee [1]. In all the cases, the callus regrew after transfer to normal culture conditions. Mineral oil dissolved 4 times the amount of the oxygen solubilized in water and supplied the minimum quantity of oxygen required for the callus to survive. Silicone dissolves 10 times the amount of oxygen solubilized in water [9]. In this paper, we investigated the possibility of long-term storage of grape 'Kyoho' callus and 'Koshusanjaku' embryogenic callus by the combination of various temperature treatments and immersion in liquid silicone.

Materials and methods

Callus of 'Kyoho' (*Vitis vinifera* L. × *V. labrusca* L.) was induced from shoot tips in vitro and subcultured monthly on the proliferation medium (Table 1), containing Gamborg's B5 basal medium [5]. Embryogenic callus that originated from the leaf disc of 'Koshusanjaku' (*V. vinifera* L.) after application of the method of Hirabayashi [6], was subcultured every 2 weeks on the proliferation medium (Table 1). Details of induction of embrogenic callus will be reported in another paper. The calli which had proliferated were used as a stock of mother cultures for all the experiments. Callus, ca 200 mg ('Kyoho') or ca 80 mg ('Koshusanjaku'), in each temperature treatment was transferred to a glass culture tube (30 × 135 mm) containing 20 ml of each storage medium (Table 1). The tubes were covered by aluminium caps, sealed with parafilm to prevent desiccation and stored in in-

Table 1. Formulation of media used for proliferation (= storage) and regeneration of grape callus.

Component	Medium		
	'Kyoho'	'Koshusanjaku'	
	Proliferation (= Storage)	Proliferation (= Storage)	Regeneration
Gamborg's B5			
inorganic elements	Full strength	Full strength	Full strength
organic elements	Full strength	–	–
Sucrose (per l)	20 g	20 g	20 g
BA	1 μM	–	–
NAA	10 μM	–	–
2,4-D	–	1 μM	–
pH	5.8	5.8	5.8
Agar (Difco Bacto)	0.7%	0.7%	0.7%

cubators at 1, 5, 10 and 15 °C in the dark. Silicone (100 Centi stokes, Wako Pure Chemical Industries, Ltd.) was autoclaved at 120 °C for 15 min and 4.5 ml added aseptically to the calli (90 mm depth). The calli were stored at 10 and 15 °C in the dark. For both cultivars, 21 to 30 tubes were tested in each temperature and silicone treatment. After the desired period of storage, the calli were weighed to determine the increase in weight during storage, transferred to fresh proliferation and regeneration medium, respectively (Table 1), and cultured at 28 °C under 16 h photoperiod regime with 3000 lux fluorescent light. The growth of callus was expressed as fresh weight increase divided by initial callus fresh weight. Calli that proliferated 2 months after the inoculation, were considered to have survived. Survival data were expressed as percentages for the number of treatment.

Results and discussion

Effects of low temperature on the storage of 'Kyoho' and 'Koshusanjaku' calli

The relative growth of 'Kyoho' calli decreased with the decrease of the temperature (Table 2). Although the growth of the calli was depressed by the preservation at 1 and 5 °C for 360 days compared with 10 and 15 °C, the calli could not survive, probably due to the damage caused by low temperature. None of the calli survived at 15 °C for 360 days, although 88% of calli were able to survive after storage for 180 days at 15 °C (data not shown). The failure at this temperature was attributed to vigorous growth and depletion of the nutrients in medium. The calli of 'Kyoho' stored at 10 °C grew slowly, and all of them remained viable after 360 days of storage. However, the calli became slightly brownish.

In 'Koshusanjaku', storage temperature affected the growth and plant regeneration from calli (Table 3). The weight of the calli stored at 15 °C for

Table 2. Effects of low temperature and silicone treatment on survival of 'Kyoho' callus for the storage of 360 days.

Treatment	Storage temperature							
	1 °C		5 °C		10 °C		15 °C	
	S[1]	G[2]	S	G	S	G	S	G
Control	0	1.2 ± 0.2	0	1.6 ± 0.5	100	48.0 ± 19.0	0	64.1 ± 26.9
Silicone	–	–	–	–	16	2.4 ± 1.4	95	57.8 ± 15.2

[1] Survival (%)

[2] Ratio of the increase over initial callus weight during storage

Table 3. Effects of low temperature and silicone treatment on growth and plant regeneration of 'Koshusanjaku' callus for the storage of 360 days.

Treatment	Storage temperature							
	1 °C		5 °C		10 °C		15 °C	
	S[1]	G[2]	S	G	S	G	S	G
Control	0	2.1 ± 0.5	4	1.5 ± 0.4	92	3.3 ± 2.3	81[3]	15.0 ± 5.5
Silicone	-	-	-	-	96	5.5 ± 2.5	100	14.9 ± 5.1

[1,2] See footnotes Table 2

[3] 88% of these calli had already regenerated during storage

360 days increased to about 15 times compared with the initial fresh weight, and only increased to about 1.5–3.3 times at 1, 5 and 10 °C. Plants could not be regenerated from calli stored at 1 and 5 °C. Clear differences in the developmental stages of the calli were observed between calli stored at 10 and 15 °C. Plants regenerated from 81% of the calli stored at 15 °C, but 88% of these calli had already developed into plants during the storage. These results suggest that plant regeneration is impaired by long storage of calli at 15 °C. In contrast, calli stored at 10 °C for 360 days did not develop into plants during the storage, and 92% of them regenerated plants only after transfer to hormone-free medium. However, the calli stored at 10 °C began to turn brownish.

Moderate depression of growth, which is important for minimum growth during the storage period, can be obtained by reduction of the temperature during growth. The most suitable temperature and frequency of subculturing in each species have been determined for the preservation of shoot tip cultures. Galzy [4] reported that conservation at 9 °C was suitable for shoot tips of grape cultured in vitro. However, our method needs further improvement for storage purposes because the calli stored at 10 °C began to brown.

Effects of silicone treatment on the storage of 'Kyoho' and 'Koshusanjaku' calli

The effect of the silicone treatment was examined in order to prolong the longevity of calli by preventing the growth and the change of color. Although 'Kyoho' calli stored at 15 °C under a silicone layer grew as well as those in the absence of silicone layer (control), 95% of the calli survived for 360 days and did not turn brownish (Table 2). In contrast, the growth of the calli at 10 °C was strongly depressed, presumably due to the strong inhibition of growth associated with a low oxygen concentration in addition to the low temperature, and only 16% of the calli were able to survive after 360

days of storage. Thus, it appeared that the suitable temperature for storage was changed by the addition of silicone.

The growth of 'Koshusanjaku' calli stored at 10 and 15 °C under silicone treatment showed the same pattern as that of control calli (Table 3). Plants never regenerated during the storage of calli either at 10 or 15 °C under silicone treatment, and plants regenerated only after transferring the calli to the regeneration medium. In contrast, plants regenerated during the storage in control calli stored at 15 °C. In addition, the calli stored at 10 or 15 °C under silicone treatment did not turn brown compared with those of the control. These results indicate that the silicone treatment prolongs the longevity of calli with a potential for plant regeneration.

In general, mineral oil has been used for minimum-growth storage of plant tissue cultures [3]. Mineral oil prevented the callus cultures from coming directly in contact with oxygen. Although mineral oil effectively depressed the calli growth, the calli turned brown and died even after 90 days of storage (data not shown). Silicone dissolves 10 times the amount of oxygen solubilized in water [9]. The regulation of the oxygen supply to the callus tissues induced by the silicone treatment may be one factor inhibiting callus growth.

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