Recovery Growth of Plants Cryopreserved by Encapsulation-Vitrification

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In vitro shoot tips of potato(14 cvs.), strawberry(6 cvs.), mint(3 species), lily(19 cvs.) and Chinese yam(3 species)were successfully cryopreserved by encapsulation vitrification. Cryopreserved shoot tips of those crops grew rapidly and vigorously *in vitro* and developed shoots directly from the meristematic area without intermediary callus formation. Their growth in the field was compared with those of control plants(same treatments other than the liquid nitrogen treatment)and no differences were detected in their growth and in the coefficiencies of variation of some plant characters. No distinct differences were observed in RAPD analysis using 200 primers between cryopreserved and control plants. This study suggests that cryopreserved plants by encapsulation vitrification are genetically stable.

Introduction

Cryopreservation has been recognized as a practical and efficient tool for long-term storage of vegetatively propagated plant germplasm with minimum space and maintenance requirements. The number of plants and varieties successfully cryopreserved has been increased sharply in the recent years since the developments of valuable and simple cryogenic procedures, such as vitrification(Sakai et al. 1990; Langis et al. 1990), encapsulation vitrification (Matsumoto et al. 1995a) and encapsulation dehydration(Dereuddre et al. 1991).

Touchell and Dixson(1995)and Benson et al. (1996) reported that encapsulation-dehydration and vitrification produced higher rates of shoot formation and recovery growth after liquid nitrogen treatment compared with the conventional slow freezing method. It is also reported that vitrified or encapsulated vitrified shoot tips following optimal treatments for each plant produced much higher rates of shoot formation and recovery growth than those of encapsulated dehydrated shoot tips of wasabi(Matsumoto et al., 1994; 1995a), lily(Matsumoto et al., 1995b), strawberry(Hirai et al., 1998), potato and mint (Hirai and Sakai, 1999a, b). Therefore, encapsulation vitrification has been applied to vegetatively propagated crops at Hokkaido Plant Genetic Resources Center since 1996, successful cryopreservation of strawberry(6 cvs.), potato (14 cvs.), mint(3 species), lily(19 cvs.)and Chinese yam(3 species)by encapsulation vitrification was established in 1999.

In cryopreservation, it is most important that cryopreserved shoot tips produce plants identical to the non-treated plants in the phenotypic aspect. Harding(1996)reported that not only phenotypic but also cytological, biochemical and molecular level analyses were necessary to confirm genetic stabilities of cryopreserved plants. There are many reports that no morphological abnormalities were occurred in cryopreserved plantlets *in vitro* but there are few reports about their growth ex situ, in the field.

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We confirmed in this study that cryopreserved shoot tips of potato, strawberry, mint, lily and Chinese yam developed shoots vigorously and directly from the meristematic area without morphological abnormalities. To assess the genetic stabilities further, a small field experiment and RAPD analysis were executed. No differences between cryopreserved and control plants were observed in their growth in the field and in the results of RAPD analysis using 200 primers. Thus, we report the genetic stabilities of cryopreserved plants by encapsulation vitrification.

Materials and Methods

Cryopreservation procedures: Potato(*Solanum tuberosum* L.),strawberry(*Fragaria x ananassa* Duch.), mint(*Mentha spicata* L.), lily(*Lilium* spp.)and Chinese yam(*Dioscorea* spp.) were cryopreserved by encapsulation vitrification. The conditions of preconditioning, preculture, osmoprotection and duration time of dehydration with PVS2 solution were described in Table 1(Hirai, 2001).

Recovery growth of cryopreserved shoot tips: The Growth of cryopreserved shoot tips was observed by microscopically from just after rewarming to the complete development of shoots. Rooted plantlets other than potato were removed from the plastic dishes and transplanted into plastic cases (about 18㎝ x 12㎝ x 5㎝) containing vermiculites. They were acclimated to ambient humidity for 2 - 4 weeks in the laboratory and planted in the field on May 5, 1997 (strawberry, 6 cvs.) or June 22, 1999 (lily, 4 cvs., Chinese yam, 1 cv.). Potato (1 cv.)plantlets were transplanted to a high sucrose concentration media (MS medium containing 0.5 g/l casamino acid, 100g/l sucrose and 2.5/l gelrite) and microtubers were induced after a month at 23 ℃ in the dark. Harvested microtubers were cold acclimated at 4 ℃ for 4 weeks and planted in plastic pots containing standard soil mix, and then, incubated for a month in the green house. They were transplanted in the field on June 22, 1999, along with non-treated (control) plantlets,

Table 1 Optimal conditions of encapsulation - vitrification method for all crops tested in this study.

Crop name (cultivar)	Preconditioning	Preculture	Osmoprotection (25℃)	PVS2 (0℃)	No.of tested cultivars
Potato (Danshakuimo)	None	0.3M sucrose (23℃, 16h)	2M glycerol+ 0.6M sucrose(+PGRs) (90min)	3h	14
Strawberry (Kitaekubo)	Cold hardening (4℃, 2weeks)	0.088M sucrose (23℃, 16h)	2M glycerol+ 0.4M sucrose (60min)	2h	6
Mint (Spearmint common)	Cold hardening (4℃, 2weeks)	None	2M glycerol+ 0.4M sucrose (60min)	3h	3
Lily (Hakugin)	Cold hardening (4℃, 3weeks)	0.088M sucrose (23℃, 16h)	2M glycerol+ 0.8M sucrose (90min)	4h	19
Chinese yam (Nagaimo,tokachi)	0.3M sucrose (25℃, 7-10d)	None	2M glycerol+ 0.6M sucrose (+PGRs) (90min)	4h	3

1) PGRs stands for plant growth regulators.
Potato : 1㎎/1 GA_3, 0.01㎎/1 BAP, 0.001㎎/1 NAA, Chinese yam : 0.2㎎/1 BAP, 0.01㎎/1 NAA

which had the same treatment plants other than cooling in liquid nitrogen.

Field measurements: Plant height was measured after harvesting(strawberry), 98 days after planting for potato and lily or 118 days for Chinese yam. All fruits (strawberry) or tubers (potato) were harvested and their numbers and total weights of fruits or tubers per each plant were measured. Plant heights, fruits or tuber weights were compared by using coefficiency of variation to detect significant differences in cryopreserved and control plants.

RAPD analysis : Genomic DNA was isolated using an ISOPLANT kit (Nippon Gene, Tokyo, Japan) from every cultivar of cryopreserved and non-treated(control)plants. DNA amplifications using 200 primers of 10 bases each (Operon technologies, CA, USA) were performed according to Williams et al.(1991). Differential bands were detected by staining with SYBR Green I nucleic acid gel-stain.

Result

Microscopic observations

Fig.1 shows periodical growth of cryopreserved potato (cv. Danshakuimo) shoot tip just after rewarming. Newly developed shoot appeared 3 days after rewarming and grew normally from the meristematic area, a complete plantlet developed within 14 days. There was no intermediary callus formation and newly regenerated shoots were observed. All shoot tips of all other plants also grew in a similar manner to potato (data not shown).

Microtuber

Microtubers were induced from both cryopreserved and control plants on sucrose enriched media and no differences in average weight and its coefficiency of variation were observed(Table 2). After cold acclimation, they started sprouting uniformly in plastic pots and grew normally in the field after transplanting.

Growth in the field

Mint and cassava could not be planted in the field because of field and climate problems. Other plants were successfully acclimatized and the mortality was under 5% in all plants transplanted in the field. There were no differences in morphological features and growth characteristics between cryopreserved and control plants (Fig. 2). The potato produced tubers within three months and strawberry produced fruits one year following transplanting.

Fig. 3 shows the average plant heights and their coefficiency of variation. Table 2 and fig.4 also show the average weight of potato tubers and strawberry fruits and their coefficiency of variation. Cryopreserved and control plants exhibited the same growth in all plant cultivars tested in this study. Thus, there were no distinct differences in average plant heights, fruits weights and tuber weights and their coefficiency of variation.

No differences in RAPD analysis were detected between cryopreserved and control plants for the 200 primers used in this study (data not shown).

Table 2 Average weights of microtubers and tubers developed from cryopreserved(+LN) and control (−LN) plants and its coefficiency of variation.

	Microtuber		Tuber	
	Average weight (mg)	Coefficiency of variation(%)	Average weight (g)	Coefficiency of variation(%)
+LN	231.3	77.3	25.0	45.7
−LN	262.4	75.4	31.6	54.7

Discussion

Shoot tips are considered as the optimal material for long-term preservation because they are genetically stable, capable of regeneration into complete plants without a callus phase and are tolerant to any formation of dehydration. Sufficiently dehydrated shoot tips are vitrified upon rapid cooling in liquid nitrogen and glassy solid is very stable below the glass transition temperature (Tg, Tg of PVS2 solution is -118 ℃). Thus, the sources of variation in cryopreserved germplasm are, 1. Insufficient dehydration of meristematic area causes lethal intracellular freezing. 2. Cells are killed by ice crystals while rapid cooling or rapid warming. 3. Shoots are regenerated from surviving cells directly or from the intermediary callus.

In this study, shoot tips of each cryopreserved plant grew rapidly and vigorously without any callus formation. It was also confirmed that all shoots were developed directly from meristematic area without any morphological abnormality. Thus, the risks of variations were almost completely eliminated by appropriate conditions of preconditioning, preculture, osmoprotection and dehydration with PVS2 solution.

Harding (1996) reported that cryopreserved potato plants growing in the glasshouse showed significant variation in plant heights compared with control groups. However those shoot tips cryopreserved by the ultra-rapid freezing method (Harding and Benson, 1994) produced lower rates of shoot formation and their mode of regeneration was more complicated (many shoots per one shoot tip) than those by encapsulation-vitrification. Takahashi et al. (1996) reported that there were no differences between cryopreserved by vitrification and control shoot tips of statice regards to plant height, flowering time and other characters. The rate of shoot formation of cryopreserved statice by vitrification was 76% and direct regrowth of shoots from the meristematic area was observed. In this study, phenotypic variations of cryopreserved potato, strawberry, lily and Chinese yam were also investigated in the field. The average rate of shoot formation of the four crops was 64.4% and no significant differences were detected in their coefficiency of variation of plant height, tuber weight of potato and fruit weight of strawberry (data not shown).

The phenotypic analysis of plants is one way to detect the variations but there are other approaches to confirm the genetic stability of plants regenerated from *in vitro* culture, such as biochemical, cytological and molecular level analyses (Harding, 1996). Recently, reagents for the isolation of DNA from plants and for PCR could be easily obtained as a whole kit and RAPD analysis became an easier way to detect the molecular level variations of plants. There are several reports to confirm the genetic stability of plants regenerated from cryopreservation by molecular analysis (Schäfer-Menuhr et al., 1996). We also found no differences in the results of RAPD analysis between cryopreserved and control potato, Chinese yam and lily. Although, RAPD analysis has its limitations and sources of error, the genetic stability of cryopreserved plants should be confirmed by other methods such as Southern blotting or nuclear-chloroplast methods (Harding & Benson, 2000).

Shoot tips cryopreserved by encapsulation vitrification method used in this study produced high rates of shoot formation and fast recovery growth. They showed no differences in their growth, both *in vitro* and in the field. Therefore, encapsulation-vitrification could be a promising method for the long-term preservation of vegetatively propagated plant genetic resources.

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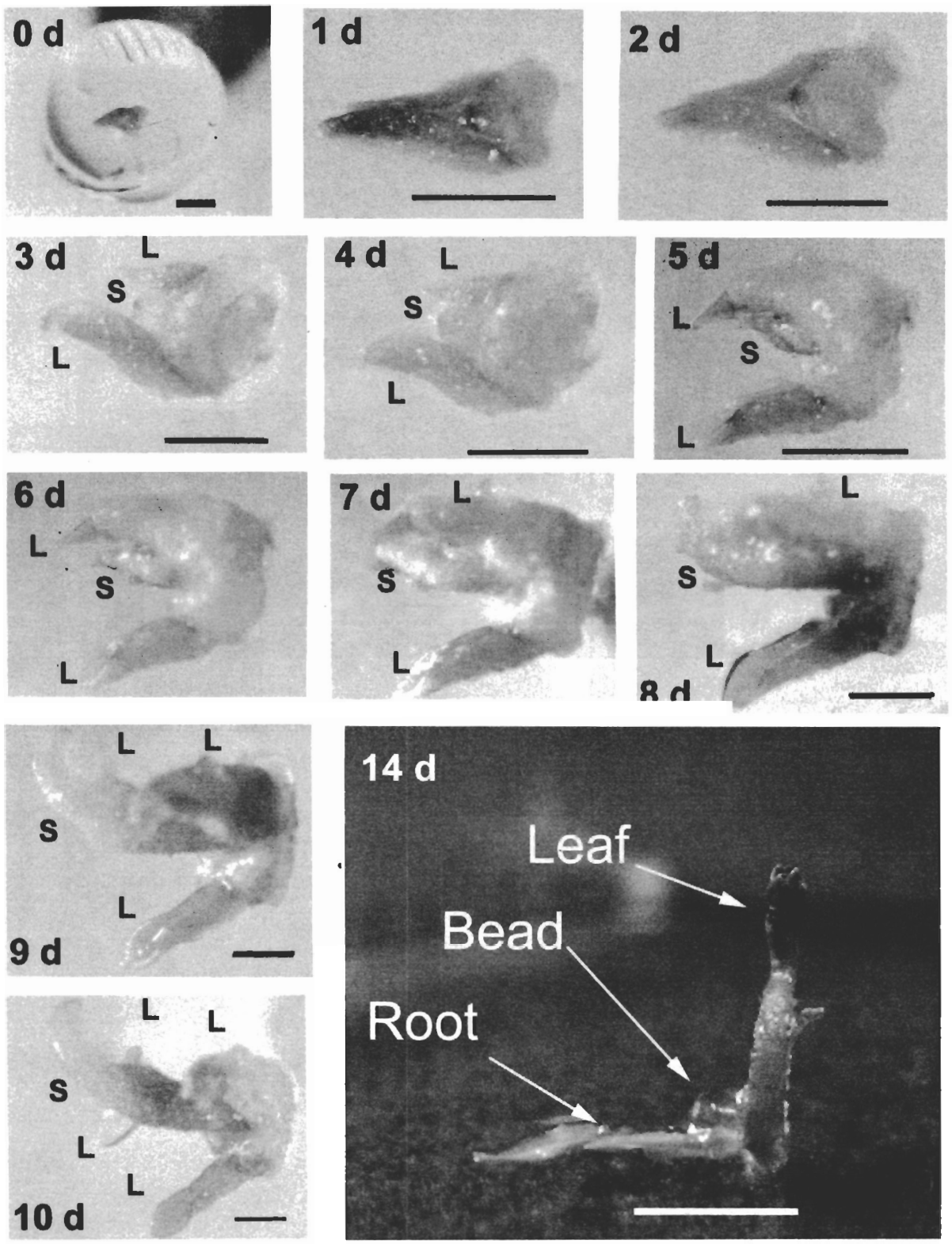

Fig. 1 Shoot formation of encapsulated and vitrified shoot tip of potato cooled to -196℃
Days on each photo indicate the period of incubation after cryopreservation.
S: shoot; L: leaf; black bars: 1 ㎜; white bar: 1 ㎝. Cultivar Danshakuimo.

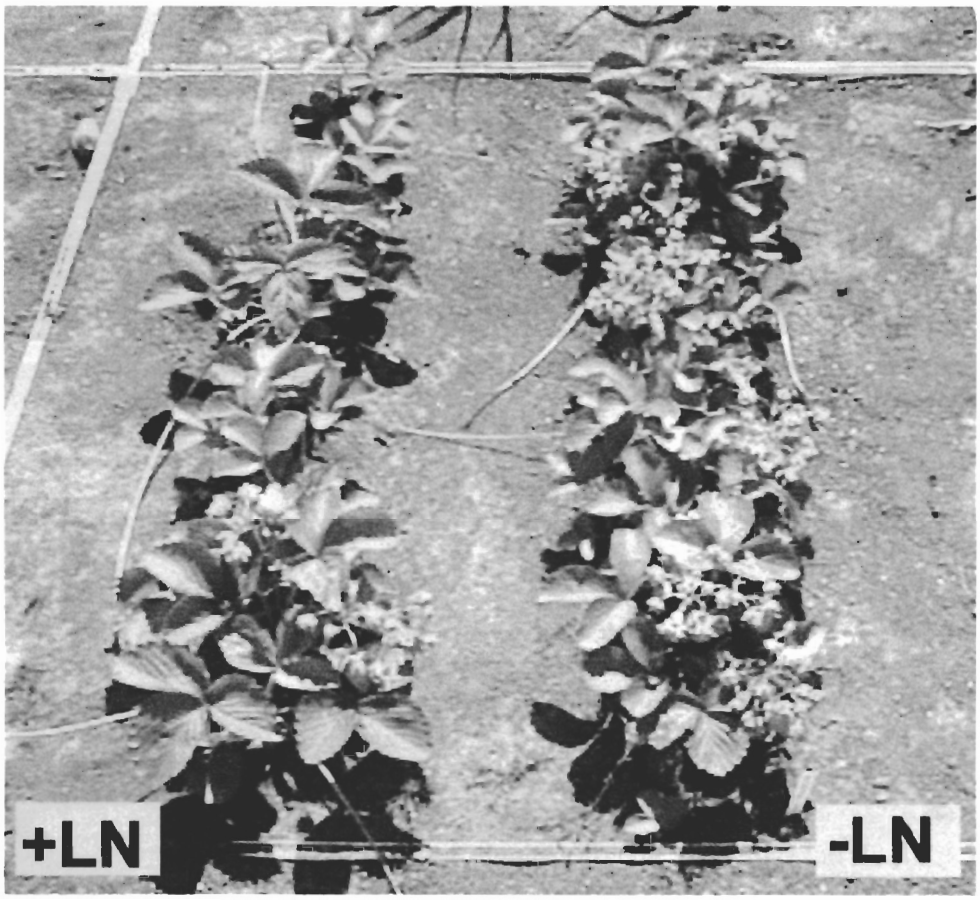

Fig. 2 Strawberry plants developed from cryopreserved by encapsulation vitrification(+LN)and control (−LN)shoot tips.
2 years after transplanting in the field. Cultivar Kitaekubo.

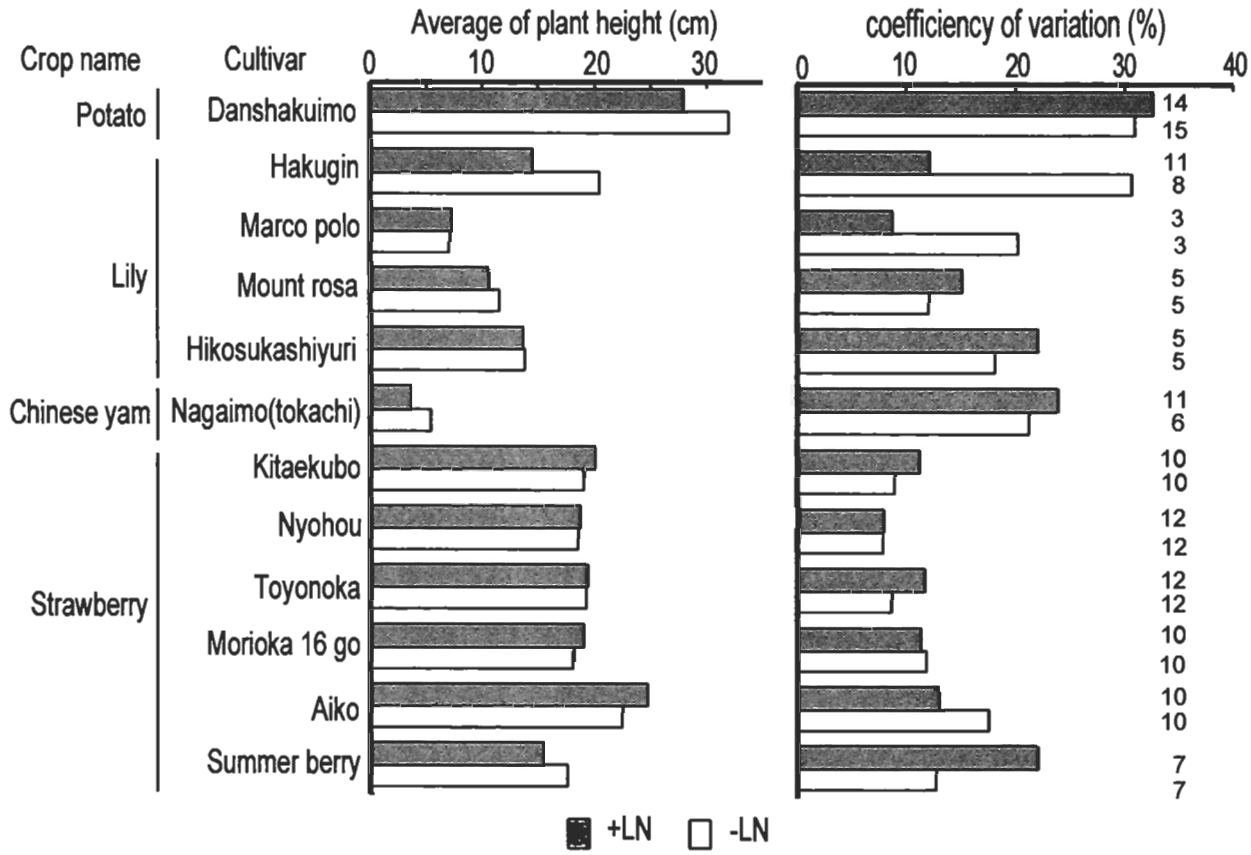

Fig. 3 Average plant height of cryopreserved(+LN)and control(−LN)plant and its coefficiency of variation.

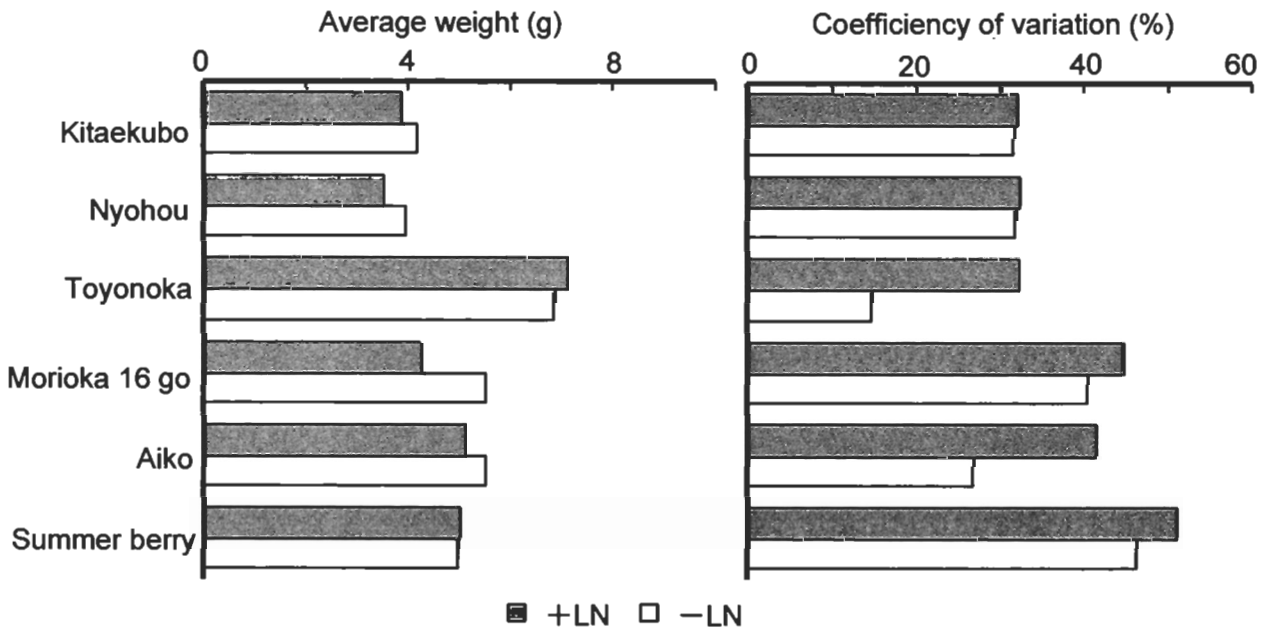

Fig. 4 Average weight of fruits developed from cryopreserved(+LN)and control(−LN)strawberry and its coefficiency of variation.

ビーズガラス化法により超低温保存した植物の生育

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要 約

バレイショ14点，イチゴ6点，ハッカ3点，ユリ19点，ヤマノイモ3点の培養茎頂をビーズガラス化法により超低温保存することに成功した。超低温保存したこれら作物の茎頂は迅速かつ旺盛に成長し，茎葉はカルスを経ることなく分裂組織から直接伸長した。圃場における生育は液体窒素処理以外は同様の処理を行った比較の植物体と差はなく，草丈などの変異係数にも差はなかった。200種のプライマーを用いたRAPD分析の結果にも明瞭な差は認められなかった。これらのことからビーズガラス化法で超低温保存した植物は遺伝的に安定していることが明らかになった。

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