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THE POST-EFFECT OF *IN VITRO* STORAGE ON SHOOT MULTIPLICATION AND ROOTING OF 'SENGA SENGANA' STRAWBERRY SHOOTS

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A B S T R A C T

'Senga Sengana' strawberry shoots were stored *in vitro* at 4ºC in the dark or at 23ºC in the light for 24 months without subculture. Every 6 months, for sampled shoots, we determined the survival and multiplication ability in three subsequent subcultures, and the rooting ability after the second subculture. The standard storage medium did not contain cytokinin or auxin. It was modified by increasing the glucose or agar concentration, or by adding mannitol or the growth retardants uniconazol or paclobutrazol. The survival rate for both the shoots stored at 4ºC in the dark for 24 months and for those stored at 23ºC in the light for 6 months was 100%. The maximal storage period at 23ºC was 12 months, which 100% or 76% of the shoots survived, depending on the experiment. The period of storage was the most important factor affecting shoot multiplication after storage under both sets of temperature and light conditions. The ability of cultures to produce axillary shoots decreased with the duration of storage, more in the first than in the second subculture, in comparison to the control cultures, which were subcultured every 5 weeks. The typical multiplication potential recovered in the third subculture. The applied storage conditions did not affect the rooting ability of shoots after the second subculture (100% of shoots rooted), or the quality and alignment of plants after acclimatization. Increasing the concentration of glucose or agar or adding mannitol to the storage medium (i.e. compounds decreasing the availability of water), or adding growth retardants did not affect the survival of shoots stored at 4°C. A higher concentration of glucose or agar, or supplementary mannitol further increased the multiplication ability of shoots stored at 4ºC for 6 months. Using CultuSac® polyethylene bags further improved the multiplication of cultures stored at 23ºC for 6 months.

Key words: agar concentration, containers, glucose, mannitol, *in vitro* storage, retardants, strawberry

Abbreviations: BAP – 6-benzylaminopurine; IBA – indole-3-butyric acid; PPFD – photosynthetic photon flux density

INTRODUCTION

In vitro technique is an optional production method for a high quality planting material of strawberry. It involves multiplying shoots arising from meristems to obtain virus-free population and their propagation by runners in insect-proof greenhouses or tents for planting in fruiting plantations. The production of micro-plants has a seasonal character. It is recommended that between seasons, cultures are stored in conditions that slow down their metabolisms, otherwise abnormal shoot tip morphology and physiological characteristics can be induced (Boxus et al., 2000). The necessity of storage in such conditions is also applicable to maintain gene banks, which are in many cases alternative ones to field collections (Reed, 1991). *In vitro* experiments on long-term storage focused rather on the survival of explants; much less concern was given to the capability for multiplication and rooting after storage. Elucidating this could help in the planning of amount of shoots to be stored.

In this paper, we present the results of experiments on the storage of shoots of the strawberry cultivar ‘Senga Sengana’. It is an old cultivar but still the number one in use in the Polish food industry. It can be cultured in conditions of sustained agriculture. For the experiments, shoots were maintained in two regimes differing in terms of temperature and light, in two different types of container, in conditions of limited water and nutrient availability, and with growth retardants added to the media. The result of 2 years of storage was characterized by the capability of the shoot to survive, the shoot quality, the ability of the shoot to multiply in three subsequent passages, and the ability to root after the second passage.

MATERIAL AND METHODS

‘Senga Sengana’ strawberry shoots, 2 cm long with 2 or 3 leaves, were obtained from cultures growing on a multiplication medium containing mineral salts of Boxus’ (1974) medium, vitamins of Lloyd and McCown’s (1980), 40 g l^{-1} glucose, 6 g l^{-1} agar (Agar Granulated, Becton Dickinson), 0.5 mg l^{-1} BA and 0.5 mg l^{-1} IBA. The standard storage medium did not contain cytokinin or auxin. It was modified for the experiments by increasing the concentration of glucose or agar, or adding mannitol or the growth retardants paclobutrazol and uniconazol. The media were sterilized at 121ºC for 15 minutes. The growth retardants were filter sterilized and added to the medium when it had cooled to 50ºC. The pH of all the media was adjusted to 5.6.

The shoots, if not otherwise stated, were stored in 350 ml glass jars (5 in each jar) containing 50 ml of one of the media, at 4ºC in the dark or at 23ºC under a 16-h photoperiod, with a PPFD of 36 µmol $m^{-2}s^{-1}$. In all experiments control shoots were stored in 350 ml glass jars on standard storage medium containing 40 g l^{-1} sucrose and 6 g l^{-1} agar, without addition of growth regulators.

The jars were covered with ventilated polyethylene caps. The cultures were placed in the storage room immediately after subculturing. After 6, 12, 18 and 24 months, we sampled 25 explants from each treatment and determined the number of living shoots and their quality, defined as the extent of necrosis on a scale from 1 to 4, where 1 – stands for total shoot necrosis, 2 – necrosis covered more than half of the shoot, 3 – necrosis covered less than half of the shoot and 4 – lack of necrosis. The healthy shoot tips were transferred to a multiplication medium and placed at 23ºC under the light conditions described above. After 5 weeks, the number of new axillary shoots was counted as the first subculture. Subsequently, 25 new shoots were put on the multiplication medium, and after 5 weeks the number of new shoots was counted as the second subculture, with the process repeated to yield the third subculture. The shoots from the second subculture were rooted on the same medium as above, but containing 0.5 mg l^{-1} IBA. Three experiments were carried out:

Experiment 1. The effect of enhanced osmolarity of the medium on the survival and multiplication of shoots stored at 4ºC or 23ºC. Media with a doubled amount of glucose, the concentration of agar increased to 9 g l^{-1}, or the addition of 10 g l^{-1} mannitol, were used. The values of the osmotic pressure in miliosmol/kg were 515, 256, 311 and 256 (control), respectively.

Experiment 2. The effect of growth retardants on the survival and multiplication of shoots stored at 4ºC or 23ºC. Media supplemented with 2 or 4 mg l^{-1} uniconazol and 2 or 4 mg l^{-1} paclobutrazol were used. The control medium did not contain growth retardants.

Experiment 3. The effect of the container type on the survival and multiplication of shoots stored at 4ºC or 23ºC. Shoots were placed in glass jars in groups of five, or singly in CultuSack® polyethylene bags (Reed, 1991).

Random design was used for all the experiments. An experimental unit consisted of a glass jar with five shoots, or five bags with single shoots. The first and third experiments were two-factorial (time period and treatment) and the second experiment was three-factorial (time period, and kind and concentration of retardants). The data were analyzed by ANOVA, and for significance estimation, $LSD_{0.05}$ was used. The Tables show the mean numbers of axillary shoots obtained from a given shoot stored for a different period of time in two subcultures on the multiplication medium.

RESULTS AND DISCUSSION

Non stored, control cultures of 'Senga Sengana' subcultured seven times every 5 weeks on the multiplication medium showed a mean multiplication rate of 3.1±0.9. The capacity for rooting of shoots obtained in the second subculture after storage was not affected by the experimental factors. All the shoots obtained in the second subculture rooted on the medium containing 1 mg l^{-1} IBA, and after acclimatization in the greenhouse, they did not show any morphological deviations.

Storage at 4ºC

All the shoots, except one, survived storage. The shoot etiolation and elongation and the extent of shoot necrosis increased with the time of storage. All the non-necrotic shoot tips became green and started to grow and produce new shoots after being transferred to fresh multiplication medium and exposed to light. The duration of storage had a significant influence on the multiplication rate, lowering it more in the first than in the second subculture (Tab. 1-3). In the third subculture, multiplication recovered to the level of the non-stored control.

Water availability regulated by an increase in the concentration of glucose or agar or by the addition of mannitol did not affect the survival or the ability to multiply of shoots stored for 12, 18 and 24 months in two first subcultures. In fact, these factors increased the multiplication ability of shoots stored for 6 months (Tab. 1). Paclobutrazol decreased the number of shoots produced during storage and the multiplication ability, especially in the first subculture (Tab. 2). Immediately after storage, the length of shoots stored on the medium with uniconazole was half as long as the control shoots, and that of the shoots on the medium with paclobutrazol was one-quarter as long. Storage in polyethylene CultuSack® bags did not affect the shoot quality after storage, but decreased the number of shoots obtained in the two subcultures (Tab. 3).

Storage at 23ºC

The longer the period of storage, the more the lower leaves had yellowed and then dried. Generally, the original shoots survived, whereas axillary shoots that had formed during storage died. At this temperature, all the shoots survived only the 6-month long storage, with the exception of those treated with mannitol (Tab. 4-6). A prolonged period of storage decreased all the parameters. As with storage at 4ºC, less axillary shoots were produced in the first than in the second subculture.

A higher agar concentration increased shoot survival in comparison to the typical concentration, and a higher concentration of glucose caused less of a decrease in the ability for multiplication (Tab. 4). The addition of mannitol reduced the number of shoots that survived 6-month storage by 20%, and made the survival of shoots stored for 12 months impossible. It also completely retarded shoot multiplication in the first subculture and caused their total necrosis in the second.

T a b l e 1 . The influence of the glucose or agar concentration in the medium, or the addition of mannitol to the medium on the number of axillary shoots of 'Senga Sengana' obtained after two subcultures after storage at 4°C in the dark (mean value from the container)

Medium composition	No. of axillary shoots obtained from stored explant			
	6 months	12 months	18 months	24 months
Glucose 40 g l^{-1}, agar 6 g l^{-1}	9.4	6.2	5.1	6.7
Glucose 80 g l^{-1}, agar 6 g l^{-1}	13.8	8.5	5.7	6.6
Glucose 40 g l^{-1}, agar 9 g l^{-1}	12.8	6.0	6.4	7.4
Glucose 40 g l^{-1}, agar 6 g l^{-1} mannitol 10 g l^{-1}	16.0	8.4	5.5	6.2
LSD 4.34				

T a b l e 2 . The influence of growth retardants on the number of axillary shoots of 'Senga Sengana' obtained after two subcultures after storage at 4°C in the dark (mean value from the container)

Medium composition	No. of axillary shoots obtained from stored explant			
	6 months	12 months	18 months	24 months
No. growth retardants	7.5	7.4	5.1	5.9
Uniconasole 2 mg l^{-1}	8.6	7.2	5.4	6.3
Uniconasole 4 mg l^{-1}	7.3	7.1	4.6	4.9
Paclobutrazol 2 mg l^{-1}	5.7	5.8	3.9	4.5
Paclobutrazol 4 mg l^{-1}	5.0	5.1	2.7	3.4
LSD 1.85				

T a b l e 3 . The influence of the type of container on the number of axillary shoots of 'Senga Sengana' obtained after two subcultures after storage at 4°C in the dark (mean value from the container)

Type of container	No. of axillary shoots obtained from stored explant			
	6 months	12 months	18 months	24 months
Glass jars	7.3	8.9	6.5	3.8
CultuSac®	5.4	6.4	4.6	3.0
LSD 2.36				

Table 4. The influence of an increase in the concentration of glucose or agar, or the addition of mannitol on the survival and number of axillary shoots of 'Senga Sengana' obtained after two subcultures after storage at 23ºC in a 16/8 photoperiod (mean value from the container)

Medium composition	% of survival		No. of axillary shoots obtained from stored explant	
	6 months	12 months	6 months	12 months
Glucose 40 g l^{-1}, agar 6 g l^{-1}	100	75	5.8	4.2
Glucose 80 g l^{-1}, agar 6 g l^{-1}	100	70	7.4	3.6
Glucose 40 g l^{-1}, agar 9 g l^{-1}	100	90	6.5	3.1
Glucose 40 g l^{-1}, agar 6 g l^{-1} mannitol 10 g l^{-1}	80	0	1.0	0
			LSD 1.65	

Retardants did not affect shoot survival, but decreased the quality of shoots stored for 6 months, and reduced the number of shoots after storage and multiplication in the first and second subcultures (Tab. 5). Nevertheless, uniconazol enabled the survival of a limited number of shoots stored for 18 months. Paclobutrazol retarded the elongation of shoots stored for 12 months, whereas the effect of uniconazole was observed after only 6 months of storage.

The storage of shoots in polyethylene bags decreased the survival of shoots stored for 12 months, and the quality of explants. The shoots stored in bags produced a higher number of shoots (in the first subculture) than those stored in jars (Tab. 6).

The results presented here confirm earlier reports (Mullin and Schlegel, 1976; Reed, 1991, 1992) that strawberry shoots can survive *in vitro* at 4ºC in the dark without subculturing for at least two years. We can add to this that shoots of strawberry can be also maintained on a medium without cytokinin and auxin in 23ºC in the light for 12 months. In both storage variants, the decrease in the multiplication ability in two subsequent subcultures was statistically significant. The full multiplication ability of the genotype recovered in the third subculture. The multiplication potential of cultures stored for 6 months at 4ºC can be improved through an increase in the glucose or agar concentration or the addition of mannitol to the storage medium. The storage of shoots at 23ºC for 12 months was more effective in CultuSac® than in glass jars.

Several factors can influence the ability of shoots to multiply after storage. Short-term storage in the cold can increase regeneration ability in comparison to the unstored control due to completion of bud dormancy, as reported for *Prunus armeniaca*

Table 5. The influence of growth retardants on the survival and number of axillary shoots of 'Senga Sengana' obtained after two subcultures after storage at 23°C in a 16/8 photoperiod (mean value from the container)

Medium composition	% of survival			No. of axillary shoots obtained from stored explant		
	6 months	12 months	18 months	6 months	12 months	18 months
No. growth retardants	100	100	0	7.1	6.1	-
Uniconasole 2 mg l^{-1}	100	100	12	9.6	5.5	-
Uniconasole 4 mg l^{-1}	100	100	8	6.4	4.3	-
Paclobutrazol 2 mg l^{-1}	100	100	0	4.9	4.1	-
Paclobutrazol 4 mg l^{-1}	100	100	0	5.1	3.8	-
				LSD 3.3		

Table 6. The influence of the type of container on the survival and number of axillary shoots of 'Senga Sengana' obtained after two subcultures after storage at 23°C in a 16/8 photoperiod (mean value from the container)

Type of container	% of survival		No. of axillary shoots obtined from stored explant	
	6 months	12 months	6 months	12 months
Glass jars	100	100	5.6	4.2
CultuSac®	100	64	11.4	7.1
			LSD 4.4	

stored for 4.5 months at 3ºC (Pérez-Tornero et al., 1999) and for shoots of *Prunus virginiana* and *Amelanchier alnifolia* stored at 4ºC for 3 months (Pruski et al., 2000). The regeneration ability of shoots stored in the cold and dark for longer periods can vary – it can be the same as in non-stored cultures as with oak (Romano and Martins-Loução, 1999), higher as with *Saussurea lappa* (Arora and Bhojwani, 1989), or lower as with elm (Dorion et al., 1993). The reaction of cultures to storage is genotype-dependent, as reported for apple rootstocks (Orlikowska, 1992) and cultivars (Negri et al., 2000).

A decrease in the shoot prolix-feration ability could be the result of the accumulation of abscisic acid in the stored plant tissues; this causes bud dormancy (Piola et al., 1998). It could also be due to ethylene over-production as the result of osmotic, low light and temperature stresses (Chi et al., 1991).

A decrease in the regeneration potential may be also a result of etiolation. According to Reed (2002), strawberry cultures survive in better

quality (less etiolated and necrotic) at 4ºC for 19 months with a 12-hour photoperiod than those stored in the dark, but information about after-storage multiplication ability was not given.

Storage in the light at 23ºC could decrease proliferation due to the exhausting of some nutrients from the medium and the decreasing cytokinin level in the shoots. The result of storing shoots of *Ensete vetricosum* at 15ºC for more than 15 months was a decrease in their ability to proliferate during the two first passages (Negash et al., 2001).

Experimental data on the effect of mannitol on the storage are not consistent. It was helpful for strawberry at 25ºC (Vysockaja, 1994) and potato at 6ºC (Sarkar and Naik, 1998), but decreased the survival rate and further proliferation of shoots of *Eucaliptus grandis* stored at 24-28ºC, and *Cedrus* sp. stored at 26ºC (Watt et al., 2000; Renau-Morata et al., 2006). In our experiment it increased proliferation ability of cultures stored at 4ºC and decreased the survival rate of shoots stored at 23ºC and made their further proliferation impossible.

Growth retardants were helpful in the storage of cherry (Snir, 1988), but in our experiments, they reduced only the length of shoots, and did not affect other parameters.

CultuSack® polyethylene bags recommended by Reed (1992) were in our experiments helpful only in storage at 23ºC for 6 and 12 months.

The decrease in the multiplication ability in the first two subcultures after storage was proportional to the length of the storage period, and this should be taken into consideration in planning the number of shoots to be stored and the number of subsequent subcultures to obtain the desired population of shoots. As to the factors which can improve the result of storage at 4ºC, we can recommend doubling the concentration of glucose, increasing agar concentration to 9 g l^{-1}, or adding 10 g l^{-1} mannitol to the storage medium. The results presented here could be taken in mind in the long-term storage of strawberry in gene banks, and in the keeping of plant stocks in laboratories producing microplants.

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ROZKRZEWIANIE I UKORZENIANIE PĘDÓW TRUSKAWKI ‘SENGA SENGANA’ PO PRZECHOWYWANIU *IN VITRO* W 4°C I 23°C

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STRESZCZENIE

Pędy truskawki ‘Senga Sengana’ były przechowywane *in vitro* w 4°C w ciemności lub w 23°C na świetle przez 24 miesiące bez przenoszenia pędów na świeże pożywki. Co 6 miesięcy określano przeżywalność i zdolność pędów do rozkrzewiania w trzech kolejnych pasażach, a także zdolność pędów do ukorzeniania po drugim pasażu. Standardowa pożywka do przechowywania była modyfikowana poprzez zwiększenie koncentracji glukozy lub agaru lub przez dodanie mannitolu lub retardantów wzrostu. Przeżywalność kultur przechowywanych w 4°C w ciemności przez 24 miesiące i w 23°C na świetle przez 6 miesięcy wynosiła 100%. Najdłuższy okres przechowywania w 23°C wynosił 12 miesięcy, gdy przeżywało od 76 do 100% pędów. Długość okresu przechowywania była najważniejszym czynnikiem wpływającym na rozkrzewianie pędów niezależnie od temperatury i warunków świetlnych. Zdolność kultur do tworzenia pędów bocznych obniżała się wraz z wydłużającym się czasem przechowywania, bardziej w pierwszym niż w drugim pasażu, w porównaniu z kulturami pasażowanymi co 5 tygodni. Kultury odzyskiwały potencjał regeneracyjny w trzecim pasażu po przechowywaniu. Zastosowane warunki przechowywania nie wpływały na zdolność pędów do ukorzeniania po drugim pasażu (100% ukorzenionych pędów) oraz na jakość i wyrównanie pędów po aklimatyzacji. Zwiększenie koncentracji glukozy lub agaru lub dodatek mannitolu do pożywki do przechowywania (tzn. związków obniżających dostępność wody) lub dodatek retardantów wzrostu (paklobutrazolu lub unikonazolu) nie wpływały na przeżywalność kultur w 4°C. Wyższa koncentracja glukozy lub agaru lub dodatek mannitolu zwiększały zdolność do rozkrzewiania pędów przechowywanych w 4°C przez 6 miesięcy. Zastosowanie torebek polietylenowych CultuSac® wpłynęło pozytywnie na rozkrzewianie kultur przechowywanych w 23°C przez 6 miesięcy.

Słowa kluczowe: koncentracja agaru, pojemniki, glukoza, mannitol, przechowywanie *in vitro*, retardanty, truskawka