ACTA SCIENTIARUM POLONORUM

Acta Sci. Pol. Hortorum Cultus, 17(3) 2018, 147–156

www.acta.media.pl ISSN 1644-0692 e-ISSN 2545-1405 DOI: 10.24326/asphc.2018.3.15

ORIGINAL PAPER

Accepted: 18.12.2017

MICROPROPAGATION OF STRAWBERRY (*Fragaria × ananassa* Duch.) ON CHEMICALLY STERILIZED MEDIA

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ABSTRACT

Micropropagation of strawberry (*Fragaria × ananassa* Duch.) is a widely used technique to produce plantlets in commercial horticulture. Unfortunately, in contrast to propagation based on runners, it is also an expensive method and may lead to obtaining plants with changed phenotype. In order to reduce such problems, the influence of chemical method of medium sterilization (application of commercial home bleach – ACE®) instead of autoclaving on the development of *in vitro* cultures of four strawberry cultivars ('Elsanta', 'Elkat', 'Senga Sengana', 'Polka') was examined. The presented study proved that sodium hypochlorite added into the hot medium is very effective, making aseptic environment agent. Applied in tested dose (2 cm^3 of ACE® per dm^{-3}) is not harmful to strawberry *in vitro* cultures and usually causes positive changes in their development (reduced formation of adventitious shoots and callus), except for 'Elsanta' cultivar. Thus the application of media sterilized with sodium hypochlorite is beneficial and cost effective and may be recommended both for commercial and especially amateur strawberry micropropagation.

Key words: sodium hypochlorite, axillary shoots, adventitious shoots, *in vitro* culture

INTRODUCTION

The method of micropropagation of strawberry (*Fragaria × ananassa* Duch.) was first elaborated by Boxus team [Boxus 1974]. Nowadays, it is a widely used technique to produce high quality [Rancillac and Nourrisseau 1989], healthy (free of viruses and other pests) true-to-type nursery plantlets in contrast to propagation based on runners [Haddadi et al. 2010]. Usually, as division of several weeks old strawberry cultures is embarrassing and laborious they are propagated through 'multi-apex shoots' method *i.e.* by the application as explants the 'tufts' consisted of axillary and adventitious shoots (fig. 1a–d). On the other hand distinguishing the origin of shoots is difficult because of callus grown up at the bottom of explant (fig. 1d–e). However, it is known that adventitious shoots are the main cause of hyperflowering and hyperrunnering phenomena [Jemmalli et al. 1994]. In Boxus [1999] opinion the only axillary shoots can give 'true-to-type' plants. Thus, to avoid the risk of adventitious shoots occurring spontaneously in strawberry *in vitro* cultures, the BA concentration should be decreased and the number of subcultures should be limited. Unfortunately, such approach increases the cost of micropropagation. Lit-

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wińczuk et al. [2009] proved the advantageous role of gibberellic acid (GA_3) in strawberry micropropagation. Higher than 0.5 mg dm^{-3} doses of GA_3 improved proliferation of axillary crown shoots or runners and reduced the development of adventitious shoots as well as the callus formation in cultures of strawberry 'Senga Sengana' and 'Elsanta' [Litwińczuk et al. 2009]. Sadly, as gibberellic acid is partially damaged during standard sterilization of medium by autoclaving, it has to be added in higher doses. It also raises the cost of media preparation what can account about 30–35% of total costs of micropropagation, included 50% of the cost of electricity consumed for autoclaving [IAEATECDOC 2004]. Micropropagation is then an expensive method. In order to reduce the cost of media preparation, some attempts of application other than autoclaving methods of medium sterilization have been made. Some chemical agents, as hydrogen peroxide [Snow 1985] and sodium hypochlorite [Teixeira et al. 2006] to reduce the contamination of medium, was attempted. Recently the chemical media sterilization was reported as advantageous for gerbera [Cordoso and Teixeira 2012], *Eucalyptus benthamii* [Brondani et.al. 2013], and wild orchids [Chansean and Ichihashi 2007] micropropagation protocols. It has been also demonstrated that sodium hypochlorite medium, iodine + potassium iodide, povidone-iodine were effective for eradicate microorganisms, causal agents of *in vitro* contamination, and provided completely sterile condition of solid MS medium without autoclaving for chrysanthemum nodes micropropagation protocol. Completely sterile condition of plant media provided by chemical disinfectant like merbromine solution (2%), thimerosal (0.1%) for chrysanthemum tissues [Deein et al. 2013] and sodium hypochlorite higher than 0.005% solution for gerbera tissues, confers toxicity [Teixeira et al. 2006].

To our best knowledge, none of these chemicals, including sodium hypochlorite, have been applied in the case of strawberry micropropagation. Therefore we examined the influence of chemical method of medium sterilization with ACE® on the development of *in vitro* strawberry cultures to evaluate its usefulness in commercial *in vitro* propagation.

MATERIAL AND METHODS

Plant material

The main experiments were conducted on previously established *in vitro* cultures of four strawberry cultivars (*Fragaria* × *ananassa* Duch.) 'Elsanta', 'Elkat', 'Senga Sengana', and 'Polka'. Additionally, the 'Honeoye' cultures from meristems were initiated and studied on chosen media.

Culture multiplication

Culture medium consisted of MS mineral salts [Murashige and Skoog 1962] supplemented with glucose (40 g dm^{-3}) meso-inositol (100 mg dm^{-3}), McCown's vitamins [Lloyd and McCown 1980], IBA (0.1 mg dm^{-3}), BA (0.5 mg dm^{-3}), GA_3 (0.5 mg dm^{-3}). The medium acidity was adjusted to pH 5.6 and supplemented with agar (AB04, Biocorp®) (7 g dm^{-3}) before sterilization. The control medium was distributed into glass jars and autoclaved at 121°C for 25 minutes. Previously the jars were washed with detergent and rinsed with tap water followed by two times rinse with deionized water and then parched at 165°C for 2 hours. The tested medium was prepared by addition of 2 cm^3 of commercial bleach (ACE®, Procter & Gamble, contained <5% active chlorine) into 1 dm^3 of hot medium (about 90°C) after agar dissolution. Then the hot medium was apportioned into unsterile culture jars. The jars were immediately closed with transparent plastic lid to obtain sterile conditions inside. Hot medium devoid of ACE® does not provide sufficient sterility what we have observed many times.

Although the mother cultures consisted of axillary and adventitious shoots as the crown shoots the only shoots of axillary origin were used as explants. They were put on standard, autoclaved medium (control) and chemically sterilized, tested medium under laminar flow cabinet. Cultures were grown in 450 cm^3 jars, filled with medium (45 cm^3 per jar) and covered with a transparent cap. The light was provided by cool white fluorescent lamps (OSRAM) at approximately 26 μmol m^{-2} s^{-1} with a 16/8 hour day/night photoperiod. The temperature was set at 24°C. Each treatment was represented by at least 4 jars (repetitions) including

8 explants during single subculture. The experiments were carried out through three subsequent 3–4 weeks long subcultures. Thus at least 96 (4×8×3) cultures per treatment were examined. At the end of passage the number of rosettes and runners of axillary and adventitious origin, as well as callus size, were determined. The number of growing cultures was recorded also.

In vitro shoot rooting

Rooting media were prepared of half-strengthen MS mineral salts, sucrose (30 g dm^{-3}), meso-inositol (100 mg dm^{-3}), McCown's vitamins, adjusted to pH 5.6 and supplemented with agar (AB04, Biocorp) 7 g dm^{-3}. The control and tested medium was sterilized by the same method as described earlier. The rosettes of axillary origin obtained separately from cultures grown on control and tested media were transferred onto control and tested rooting media, respectively. Each treatment was represented by at least 4 jars (repetitions) including 8 explants during single subculture. The experiments were carried out through three subsequent 3–4 weeks long subcultures. Thus at least 96 (4×8×3) cultures per treatment were examined. At the end of subculture the number of rooted shoots, the number of roots per explant were recorded.

Acclimation

Rooted plantlets were transferred into peat substrate in 5 cm pots and kept in a high humidity chamber covered with a transparent lid. The ambient temperature was 26°C with 16/8 h day/night light period. After a week the lid was slightly open and after next four weeks following data were recorded: length of the middle leaflet, the width of the middle leaflet, petiole length. The relative chlorophyll content was measured using Konica Minolta SPAD 502 Plus Chlorophyll Meter.

Additional study

The experiments described below, as supplementary, were carried out on limited number of cultures and cultivars.

Meristem culture. The culture was initiated from field-grown 'Honeoye' strawberry plants, propagated by the only conventional method (through runners). Collected runners had been soaked for 24 hours in fungicide solution: Topsin M 500 SC (2 g dm^{-3}), Euparen Multi 50 WP (1 g dm^{-3}), and a drop of Tween 20. Then runner tips were excised, rinsed in tap water, dipped in 70% ethanol for 1 minute, and soaked in 20% ACE solution (with a drop of Tween 20) for 20 minutes. After surface sterilization tips were rinsed 3 times for 5 minutes with sterile deionized water. Initial explants (about 0.3–0.7 mm in diameter) contained apical meristem with two or three leaf primordia, were prepared and transferred separately into 10 cm^3 glass test tubes filled with 2 cm^3 of medium covered with aluminum foil. The media for meristem cultures consisted of half-strengthen MS mineral salts, sucrose (20 g dm^{-3}), meso-inositol (100 mg dm^{-3}), PVP 360 (1 g dm^{-3}), McCown's vitamins. Two PGR combinations were tested. The first ('3') was made by addition of IBA (0.1 mg dm^{-3}), BA (0.1 mg dm^{-3}), and GA_3 (0,1 mg dm^{-3}), the second one ('1') by supplementation with IAA (1 mg dm^{-3}). Each combination was represented by at least 20 meristems. The method of media sterilization, as well as culture conditions, were the same, as described earlier. After 4 weeks the number of viable cultures was recorded.

Chlorophyll fluorescence analysis. 'Polka' and 'Senga Sengana' *in vitro* cultures during multiplication phase (subculture) were subjected to analysis of chlorophyll fluorescence. They were performed on dark-adapted cultures. The WALZ GmBH IMAGING-PAM M-Series (MAXI version) apparatus was used. The following parameters were recorded: F_0 – minimal fluorescence yield, F_m – maximal fluorescence yield, F_v – variable fluorescence, F_v/F_m – maximal PS II quantum yield, Abs – absorptivity. Due to high possibility of culture contamination and other technical problems, like difficulties in camera focusing, fast desiccation of cultures, the measurements were done on small number of leaves (2 jars with 8 cultures for treatment).

Statistical analyses

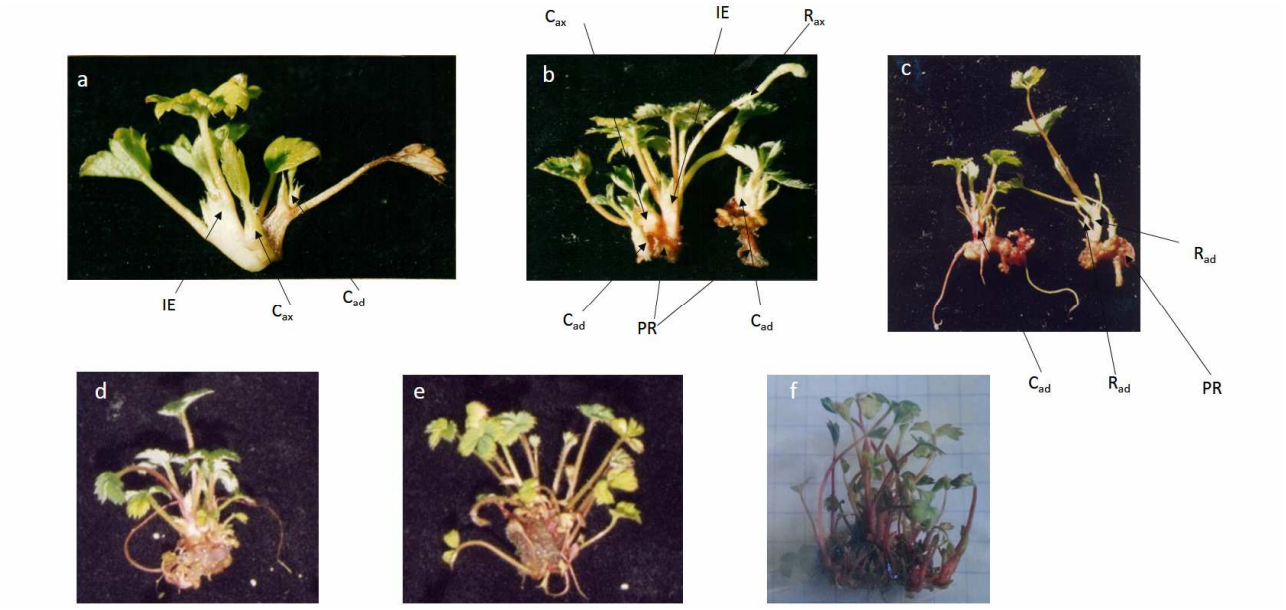
At the end of passage cultures were took out from jar and the number of axillary (AX) and adventitious (AD) shoots, as well as size of callus were determined. Then the ratio of AX shoots was calculated according to following formula: 100% × number of AX shoots/number of shoots (both AX and AD ones). Collected data were subjected to an ANOVA, LSD

mean separation test at $\alpha = 0.05$ using Statistica 9.0 computer software. Data presented as percentage (number of cultures developed exclusively AX shoots, number of rooted shoots, *etc.*) were subjected to test on difference between two proportions. Cluster analysis based on Ward's method and Euclidean distance was used to evaluate the similarity of cultures reaction on studied media. The following traits were used for agglomeration: mean number of axillary rosettes, mean number of adventitious rosettes, mean number of axillary runners, the mean number of adventitious runners, size of callus, number of rooted shoots, number of roots per explant, length and width of the middle leaflet, petiole length, relative chlorophyll content [SPAD].

RESULTS

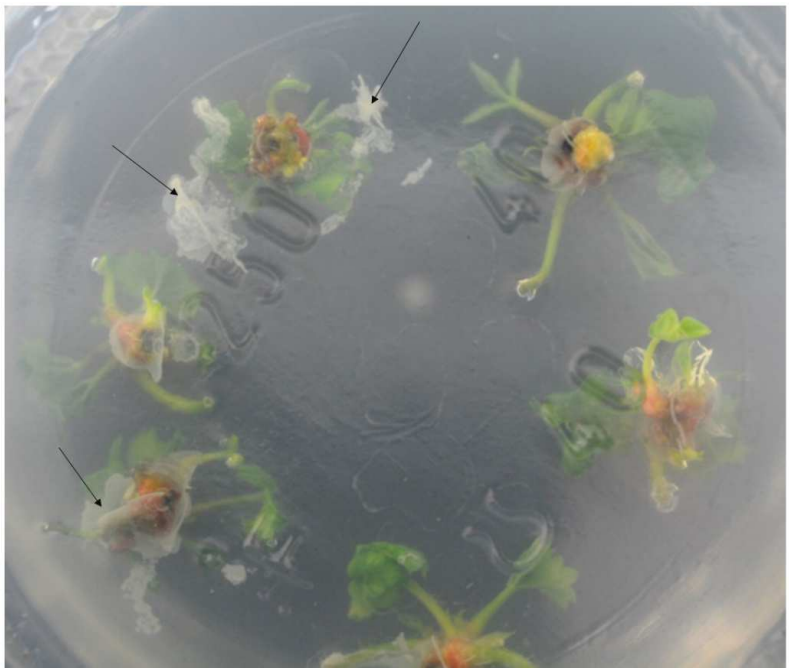
Culture multiplication

Strawberry *in vitro* cultures of every cultivar developed axillary and adventitious shoots on autoclaved (control) and chemical sterilized (tested) media. The adventitious shoots appeared at the petiole between stipules or at the petiole remains before callus expansion (fig. 1a–c). Aside from crown shoots also runner development was observed f. 1b–c). Any differences in color and shape of leaves of strawberry cultures or symptoms of physiological disorders were not found. It turned out that some strawberry cultures was settled with endophytes. Their colonies sometimes appeared on control medium (fig. 2) whereas were not observed on the chemically sterilized one. Significant differences in the reaction of studied strawberry cultivars on the tested media were found. In comparison with control, the proliferation of 'Elsanta' shoots was stimulated on the tested medium, whereas retarded in the case of 'Elkat' clone (tab. 1). A total number of shoots in cultures of 'Senga Sengana' and 'Polka' remained unchanged. All strawberry clones developed axillary and adventitious shoots both on autoclaved and chemical sterilized media. The proportions between the number of axillary (AX) and adventitious (AD) shoots were similar for both media except for 'Elkat' cultures where both relative and real adventitious shoot development was retarded on chemically sterilized medium (tab. 1).



a – two weeks old culture; b,c – three weeks old culture; d,e – callus at the base impedes distinguishing axillary shoots from adventitious shoots (after 4 weeks), f – cultures strongly dominated with adventitious shoots (after 4 weeks). IE – initial explant, C_{ax} – axillary crown (rossetes), R_{ax} – axillary runner, C_{ad} – adventitious crown shoot, R_{ad} – adventitious runner, PR – adventitious shoot on petiole remain

Fig. 1. Strawberry *in vitro* culture during multiplication stage



Arrows indicates endophytes on medium around the cultures

Fig. 2. Colonies of endophytes in strawberry 'Polka' *in vitro* cultures on the autoclaved medium

Table 1. The development of strawberry *in vitro* cultures during multiplication phase depending on medium sterilization treatment

Cultivar	Elkat		Senga Sengana		Elsanta		Polka	
Type of medium	control*	tested**	control	tested	control	tested	control	tested
Total number of shoots	7.0 d	4.2 b	5.5 c	5.6 c	3.1 a	7.6 d	5.5 c	5.5 c
Total number of axillary shoots	5.3 de	3.7 b	4.0 bc	4.4 bc	2.7 a	6.2 e	4.8 cd	4.7 bcd
Axillary rosettes	5.0 f	3.0 cd	1.5 a	2.4 bc	2.1 ab	4.2 ef	3.6 de	3.7 de
Axillary runners	0.3 a	0.7 abc	2.5 d	2.0 d	0.6 ab	2.0 d	1.2 c	1.0 bc
Ratio of axillary shoots in culture (%)	78.3 a	91.3 b	78.7 a	82.6 ab	90.8 b	87.3 b	87.9 b	89.9 b
No. of cultures consisted of the axillary shoots only (%)	43 a	75 c	56 ab	67 bc	78 c	70 bc	75 c	77 c
Total number of adventitious shoots	1. 6 b	0.4 a	1.5 b	1.2 ab	0.4 a	1.4 a	0.7 a	0.8 a
Adventitious rosettes	0.7 b	0.1 a	1.1 b	0.8 b	0.2 a	0.8 b	0.7 b	0.7 b
Adventitious runners	0.9 d	0.3 abc	0.3 bc	0.4 bc	0.2 bc	0.6 c	0.03 a	0.2 b
Size of callus [1–5]***	2.2 d	1.1 c	1.0 c	0.3 b	0.0 a	3.5 f	4.0 g	3.0 e

Means followed by different letters within a row are significantly different at p = 0.05; * autoclaved medium, ** medium sterilized with ACE, *** 1 – smaller than 2 mm, 5 – bigger than 10 mm

Cultures grown on studied media consisted of mainly axillary rosettes (crown shoots) although the axillary runners appeared also, especially in the case of 'Senga Sengana'. 'Elsanta' clone cultured on the tested medium developed significant more runners than on control (tab. 1). The tested medium changed the proportion between the number of adventitious crown shoots and runners. 'Elsanta' *in vitro* cultures produced more adventitious rosettes while 'Polka' cultures – more adventitious runners on the tested medium in comparison with control (tab. 1). Strawberry cultures formed a callus on the explant base. In comparison with control, callus growth was retarded in 'Elkat', 'Senga Sengana' and 'Polka' clones, except for 'Elsanta' one, where the adverse phenomenon was observed (tab. 1).

In vitro rooting

Some differences among cultures in the response on studied media were found (tab. 2). Contrary to 'Elsanta', the 'Polka' explants rooted better on the tested medium and produced more roots in comparison to the control. Rooting of 'Elkat' and 'Senga Sengana' shoots was similar on both media' (tab. 2). Any differences in color and shape of leaves of strawberry cultures or symptoms of physiological disorders were not found.

Acclimation

Rooted *in vitro* shoots were transferred to peat substrate and had grown for 4 weeks. Almost all plantlets (95–100%) were successfully acclimated. There were no differences in the shape and color of leaves of acclimated plants. The differences in the size of leaflets were not observed in the case of all strawberry cultivars except for 'Senga Sengana', where leaflets of plants obtained from the tested medium were smaller than control ones (tab. 3). Simultaneously the petiole length was also significantly shorter. Such effect was also observed in the case of 'Polka' plants where the ratio between the petiole and middle leaflet length was reduced (tab. 3). Some differences in the relative chlorophyll content were found. The SPAD index recorded for 'Elkat' and 'Elsanta' plants obtained from tested medium was lower than for control. In the case of other two cultivars such phenomenon was not confirmed (tab. 3).

Table 2. Rooting *in vitro* of strawberry shoots depended on medium sterilization method

	Elkat		Senga Sengana		Elsanta		Polka	
Type of medium	control*	tested**	control	tested	control	tested	control	tested
No. of rooted shoots (%)	87.5 bc	93.7 cd	100.0 d	97.1 cd	93.7 cd	75 ab	59.4 a	93.7 cd
No. of roots per explant	4.1 b	4.0 b	3.7 b	3.6 b	3.9 b	2.1 a	1.9 a	3.5 b

Means followed by different letters within a row are significantly different at p = 0.05; * autoclaved medium, ** medium sterilized with ACE

Table 3. Characteristics of acclimated plantlets

Cultivar	Elkat		Senga Sengana		Elsanta		Polka	
Type of medium	control*	tested**	control	tested	control	tested	control	tested
Length of middle leaflet (mm)	15 abc	15 ab	19 d	15 a	15 ab	15 abc	17 cd	17 bcd
Width of middle leaflet (mm)	13 ab	13 ab	15 b	12 a	13 a	14 ab	17 c	17 c
Length /width of middle leaflet ratio	1.2 b	1.2 b	1.2 b	1.2 b	1.2 b	1.1 ab	1.1 a	1.0 a
Petiole length (mm)	25 bc	22 ac	36 d	26 c	20 a	19 a	27 c	22 a
Petiole length / middle leaflet length ratio	1.7 bc	1.5 ab	2.0 d	1.8 cd	1.3 a	1.3 a	1.6 bc	1.3 a
Relative chlorophyll content (SPAD)	39 bc	34 a	42 d	40 c	38 bc	34 a	39 bc	38 b

Means followed by different letters within a row are significantly different at p = 0.05; * autoclaved medium, ** medium sterilized with ACE

Complex cultivars response to studied media

Notable differences in the reaction of studied strawberry cultivars on the studied media were found. The shoots and callus proliferation of 'Elkat' and 'Polka' both on control and tested media were similar contrary to other cultivars (fig. 3a–b). The reaction of 'Elsanta' clone, manifested by significantly increased growth of shoots and callus on ACE medium, was most distinct (fig. 3a–b, tab. 1). This cultivar was also most ACE-sensitive in subsequent stages of micropropagation (fig. 3c–d), especially during rooting shoots *in vitro* (tab. 2).

Additional experiments and analysis

Regeneration of meristems. Four media combinations were the tested during the initiation of 'Honeye' cultures from meristems (fig. 4). The relationship between meristem dieback and kind of medium was not proofed statistically. However, both in the case of media supplemented with IAA alone or supplemented with 3 PGRs (IBA, BA, GA_3) – sterilized chemically the number of meristems which started to grow was about 5% higher than recorded for control medium (38% > 35%, 50% > 43%, respectively).

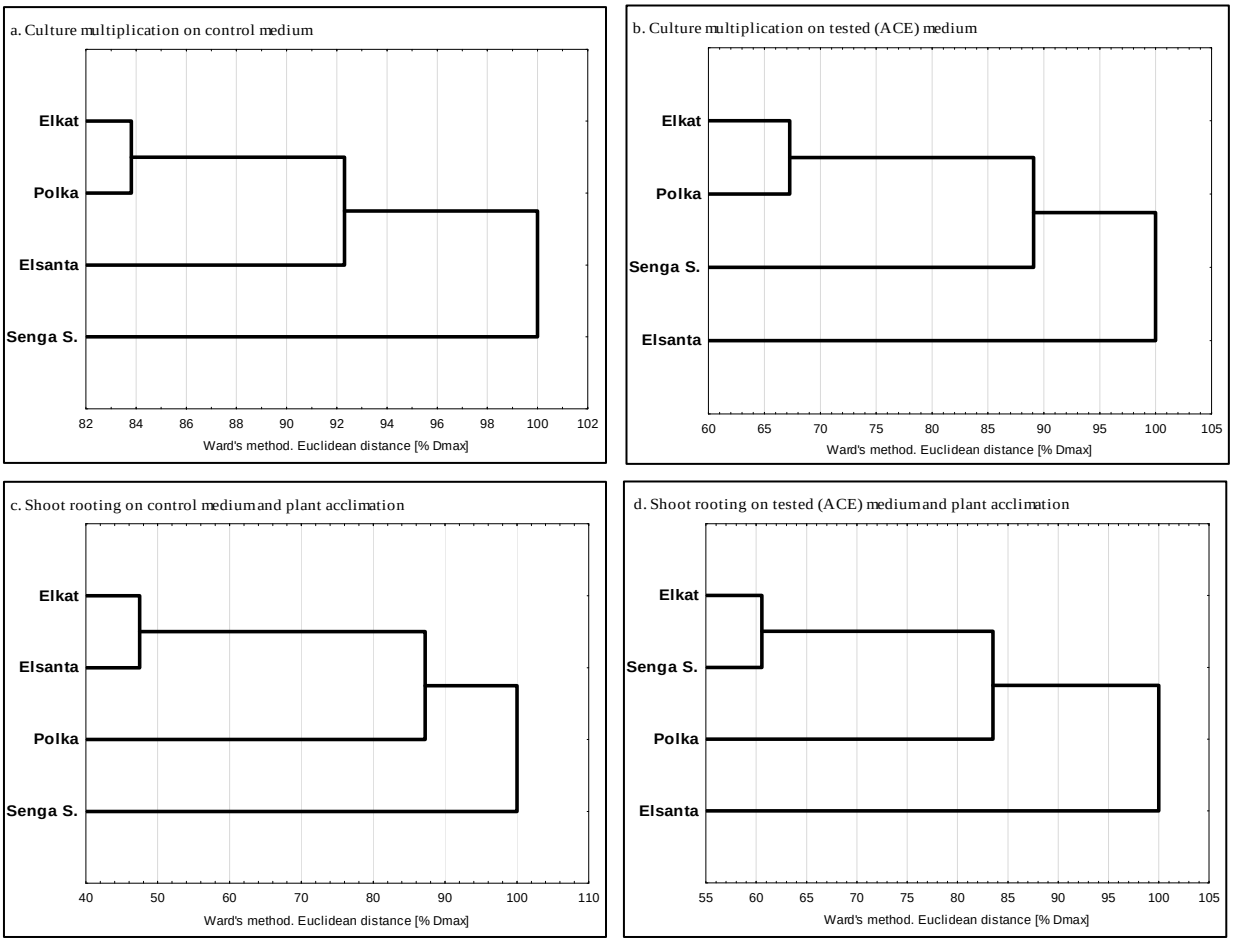
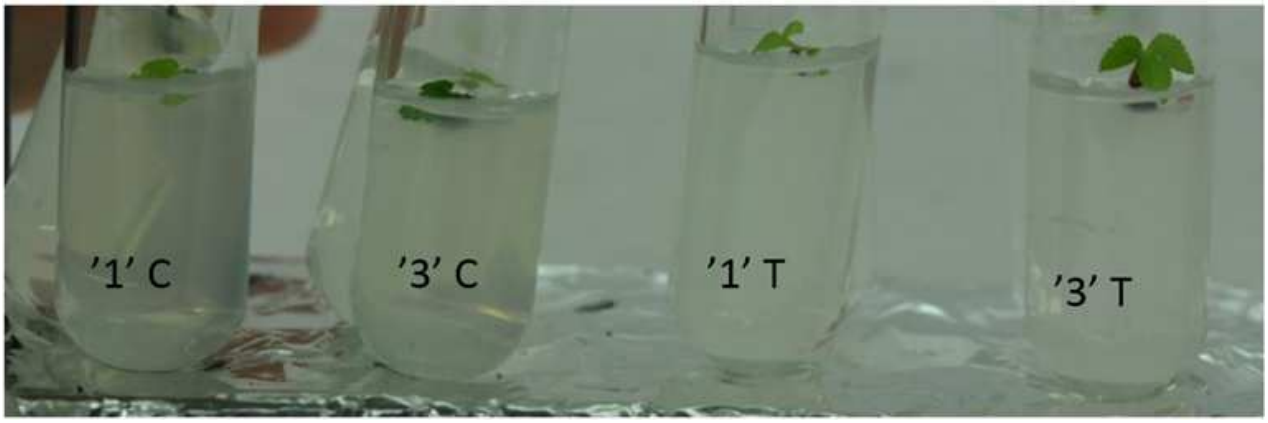


Fig. 3. The similarity of four strawberry cultivars in response to the method of medium sterilization



'1' C – control medium with 1 PGR, '3'C– control medium with 3 PGRs, '1' T – chemically sterilized medium with 1 PGR, '3'T – chemically sterilized medium with 3 PGRs

Fig. 4. Initiation stage of culture from meristem of two weeks old culture

Table 4. Chlorophyll fluorescence of 'Senga Sengana' and 'Polka' in vitro cultures during multiplication stage

Cultivar	Medium	F_0	F_m	F_v	F_v/F_m	F'_0	F'_m	F'_v	F'_v/F'_m	Abs
Polka	control*	0.245 a	0.667 b	0.435 b	0.655 a	0.243 b	0.680 b	0.438 b	0.646 a	0.929 b
	tested**	0.174 a	0.514 a	0.340 a	0.677 a	0.170 a	0.499 a	0.330 a	0.671 a	0.891 a
Senga Sengana	control	0.138 a	0.491 a	0.353 a	0.720 a	0.136 a	0.476 a	0.340 a	0.714 a	0.918 a
	tested	0.172 a	0.534 a	0.362 a	0.682 a	0.171 a	0.519 a	0.348 a	0.676 a	0.925 a

Means followed by different letters within a row are significantly different at p = 0.05; * autoclaved medium, ** medium sterilized with ACE

Chlorophyll fluorescence analysis. Chlorophyll fluorescence analysis was performed on *in vitro* cultures in multiplication stage. The differences for 'Senga Sengana' clone cultured on studied media were not found contrary to 'Polka' cultures (tab. 4). In general control F'_0, F'_m and F'_v values were higher than for 'Polka' cultures grown on tested media. The relative chlorophyll content (Abs) was also higher. On the other hand, the F_v/F_m ratios were similar (tab. 4).

DISCUSSION

Despite the method of micropropagation of strawberry is well elaborated, some problems still exist, among others the high cost of medium preparation. Autoclaving of medium significantly raises the cost and extends the operating time of its preparation [Macek et al. 1995]. Sterilization of plant cultures medium with high temperature and increased pressure causes the degradation of temperature labile components, changes the chemical composition and creates toxic products of degradation of sugars, such as furfural [Brondani et al. 2013] The medium microwaving occurred unsuccessfully and is not often used for media preparation [Teixeira and Torres 1998; Tisserat et al. 1992]. Chemical sterilization of medium may be an alternative to autoclaving. Hypochlorite is a sterilizing agent frequently used for asepsis of plant tissue because of low toxicity in comparison with other chemicals such as mercury salts, antibiotics and fungicides [Niedz and Bausher

2002; Tiwari et al. 2012]. On the other hand Teixeira et al. [2006] reported that sodium hypochlorite higher than 0.005% solution often conferred toxicity for gerbera tissues. In our previous and preliminary studies (unpublished data), we found that sodium hypochlorite (0.01%) was toxic for highbush blueberry, cranberry, mulberry, and chokeberry *in vitro* cultures. The only exception were strawberries. Thus, we assessed the influence of chemical method of medium sterilization with ACE® on development of *in vitro* strawberry cultures and evaluated its usefulness in commercial propagation. The cultures of four tested strawberry cultivars were tolerant on 0.01% sodium hypochlorite solution. However, the experiments showed distinct differences in the development of four tested strawberry cultivars with respect to the studied media, what is typical for many plant species. In the multiplication stage, the chemical sterilized medium did not have a negative effect on shoot proliferation with exception of 'Elkat'. However, in the case of this cultivar, the development of adventitious shoots was retarded much stronger than the growth of axillary shoots, which is a positive effect. Generally, cultures of other three clones growing on the chemically sterilized medium produced at least the same quantity of axillary shoots. Usually, they formed smaller callus which facilitates distinguishing axillary and adventitious shoots (tab. 1). Described effects may be caused by gibberellic acid which was not destroyed by autoclaving as similar changes in the development of strawberry *in vitro* cultures were observed by Litwińczuk et al. [2009] who used autoclaved media supplemented with high doses of GA_3 (0.5–10 mg dm^{-3}). This positive effect may be also related to the nutrient role of chlorine [Ramage and Williams 2002]. Its positive influence on shoots growth was reported by Teixeira et al. [2006] for other plant species as: *Ananas comosus*, *Eucalyptus pellita*, *Pfaffia glomerate*. On the other hand, it is also known, that presence of chloride ions (Cl^-) in the soil in higher amounts can be toxic to strawberry plants, as affecting photosynthesis [Gulen et al. 2016]. However, the results of chlorophyll fluorescence parameters did not point at the strong detrimental influence of Cl^- ions especially on multiplied 'Senga Sengana' strawberry cultures, probably due to buffering role of the medium and/or weak photosynthesis in *in vitro* conditions. On the other hand, the negative effect of chlorine was confirmed by decreased relative chlorophyll content and/or smaller leaves of acclimated plantlets. Nonetheless, the further growth of plants was not worsened (data not presented). Supplementary experiments did not prove also the harmful effect of sodium hypochlorite on *in vitro* shoot rooting (except for 'Elsanta' cultivar) as well as the development of isolated strawberry 'Honeye' meristems.

Summarizing, the presented study proved that sodium hypochlorite added into plant medium is very effective agent making aseptic environment. Applied in tested dose is not harmful to strawberry *in vitro* cultures and usually causes positive changes in their development (except for 'Elsanta' cultivar). Thus the application of media sterilized with sodium hypochlorite is beneficial and cost effective and may be recommended for commercial and especially amateur strawberry micropropagation.

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