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IN VITRO SELECTION OF *Solanum Pimpinellifolium* PLANT TOLERANT TO NaCl

SELEKCJA ROŚLIN *Solanum Pimpinellifolium* O PODWYŻSZONEJ TOLERANCJI NA ZASOLENIE W KULTURACH *IN VITRO*

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Streszczenie. Zasolenie jest jednym z głównych stresów abiotycznych na świecie ograniczających o ponad 50% plon głównych roślin uprawnych, takich jak pomidor. Odporność pomidora na zasolenie jest cechą zależną liniowo lub odmianowo. Celem badań było określenie zróżnicowania tolerancji na stres solny linii pomidora *S. pimpinellifolium.* W celu indukcji tworzenia tkanki kalusowej zastosowano 6 kombinacji roślinnych regulatorów wzrostu BAP i IAA, dodanych do pożywki MS. Największą masę tkanki kalusowej o ciemnozielonym zabarwieniu zaobserwowano na pożywce MS uzupełnionej 2,0 mg · dm^{-3} BAP i 2,0 mg · dm^{-3} IAA. Następnie zaindukowany kalus oraz eksplantaty pędowe pomidora wykładano na pożywkę MS z różną zawartością soli NaCl: 0 (obiekt kontrolny), 25, 50, 75, 100, 125 i 150 mM. Wykazano, że stres solny negatywnie wpłynął na wzrost i rozwój roślin, a obecność 125 i 150 mM NaCl w podłożu całkowicie zahamowała proces tworzenia kalusa oraz indukcję zarodków somatycznych. Wyniki doświadczenia wykazały, że somaklony *S. pimpinellifolium*, uzyskane z kalusa selekcjonowanego na pożywce MS z dodatkiem 100 mM NaCl, charakteryzowały się podwyższoną tolerancją na stres solny.

Key words: callus, salt stress tolerance, somatic embryogenesis.
Słowa kluczowe: kalus, somatyczna embriogeneza, tolerancja na zasolenie.

INTRODUCTION

Salinity is one of the main abiotic stresses which significantly reduce plant productivity worldwide (Alian et al. 2000; Flowers 2004; Goel et al. 2010; Rzepka-Plevneš et al. 2010; Rai et al. 2010; Al Hassan et al. 2014; Zaki and Yokoi 2016). Out of 1.5 billion hectares of cultivated areas around the world, about 5% shows increased concentrations of salt (Abdel Latef and Chaoxing 2011). A wide range of plant species, including the most important crops, grow in moderately saline environments. Salinity tolerance is defined as the ability of a plant to maintain growth in saline conditions (Skrumsager Møller and Tester 2007). The induction of salt tolerance in plants through conventional breeding practices is seriously limited by the complexity and polygenic nature of salinity tolerance (Rai et al. 2010; Zaki and Yokoi 2016).

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One of the methods of accelerating research on this feature and thus providing salt tolerant components for cross-breeding is screening under *in vitro* conditions (Patade et al. 2008; Krupa-Małkiewicz et al. 2015). The evaluation of salt tolerance in tissue culture can be more useful for breeding programs, because selection can considerably shorten the time, minimize environmental interaction (such as pathogens, water deficit, high/low temperature, high light intensity) and can complement field selection (Jain 2001; Krupa-Małkiewicz et al. 2015; Piwowarczyk et al. 2016; Zaki and Yokoi 2016).

Development of plants tolerant to abiotic stress, especially to salt and drought, using *in vitro* selection has been reported in reference to a wide range of plant species including cereals (Noaman 2000; Nawaz et al. 2013), vegetables (Rafiq et al. 2008), fruits and other commercially important plant species (Rzepka-Plevneš et al. 2007; Patade et al. 2008; Rai et al. 2010; Al Hassan et al. 2014). According to Rzepka-Plevneš et al. (2007), who have studied salt stress effects by exposing the callus to a different level of NaCl, it was found that the physiological and biochemical indicators play an important role in salt tolerance.

According to Zaki and Yokoi (2016) it is very important to screen the available cultivated and wild species for their salt tolerance in order to recommend cultivars that can be cultivated in high saline conditions or to use salt-tolerant genotypes in breeding programs.

The objective of this study was induction of somaclonal variability in *S. pimpinellifolium* tomato callus culture, and screening for salt tolerance plants regenerated through somatic embryogenesis.

MATERIAL AND METHODS

Plant material

The seeds of tomato *Solanum pimpinellifolium* (L0566) constituted the plant material for this study. The seeds were obtained from Tomato Genetics Resource Centre (University of California, Davis). Before germination, the seeds were soaked in sterile distilled water for 12 hours and then disinfected in two steps. In the first one, a 70% solution of alcohol was applied for 30 seconds, and in the second step, a 7% solution of hypochlorite (NaOCl) was used for 10 minutes. After that, the seeds were rinsed three times in sterile distilled water for 5 minutes per rinse. Next, the seeds were dried on sterile absorbent paper and each seed was placed into 100 ml Erlenmeyer flask filled with 20 ml of MS medium (Murashige and Skoog 1962). The flasks were incubated in growth room for the period of 14 days.

Initiation of callus culture

The explants used to initiate the callus cultures were the fragments of cotyledons and first leaves of an area of approx. 0.5 cm^2. Explants were placed on MS medium supplemented with BAP (6-benzyloaminopurine) and IAA (indole-3-actic acid) in 6 combinations (Table 1). The control were explants from MS medium without addition of plant growth regulators. After 4 weeks the initiated callus was then divided into fragments of 3 mm diameter and the weight of approx. 0.05 g, and propagated three times on MS medium supplemented with 2.0 mg · dm^{-3} BAP and 2.0 mg · dm^{-3} IAA.

Table 1. The influence of plant growth regulators on callus initiation in *in vitro* culture of *L. pimpenellifolium*
Tabela 1. Wpływ roślinnych regulatorów wzrostu na inicjację tkanki kalusowej *L. pimpenellifolium*

Plant Growth Regulators Roślinne regulatory wzrostu [mg · dm^{-3}]		Callus weight Masa kalusa [g]	Colour and structure of callus tissue Kolor i struktura tkanki kalusowej
BAP	IAA		
0	0	0.06d	light green, compact jasnozielony, zbity
1	0	0.06d	green, loose zielony, luźny
1	1	0.12c	green, losse zielony, luźny
1	2	0.13c	green, losse zielony, luźny
2	0	0.12c	dark green, compact ciemnozielony, zbity
2	1	0.23b	dark green, compact ciemnozielony, zbity
2	2	0.32a	green, losse zielony, luźny
		$LSD_{\alpha\ 0.05}$ = 0.06 $NIR_{\alpha\ 0,05}$ = 0,06	

Means in the same column followed by the same letter are not significantly different (α < 0.05; Least Significant Differences test LSD).
Średnie oznaczone tymi samymi literami nie różnią się istotnie (NIR – najmniejsza istotna różnica; α < 0,05).

Selection for salinity in callus culture

The obtained callus was divided into fragments of 3 mm in diameter and the weight of approx. 0.05 g. Clumps of 25 calli were placed on MS medium supplemented with 2.0 mg · dm^{-3} BAP and 2.0 mg · dm^{-3} IAA with the addition of NaCl salt in concentrations: 25, 50, 75, 100, 125 and 150 mM, as well as without NaCl salt addition (control). The experiment was conducted in four replications. After four weeks, the rate of callus growth on test and control media was determined.

Regeneration of tolerant form by somatic embryogenesis

The fragments of callus of approx. 3 mm in diameter and the weight of approx. 0.05 g, respectively proliferated on MS medium with addition of different concentration of NaCl salt were transferred on somatic embryogenesis MS medium supplemented with 2.0 mg · dm^{-3} NAA (napthaleneacetic acid) and 5.0 mg · dm^{-3} BAP. 100 explants were obtained from each combination of selection media. The stage of somatic embryo initiation lasted 8 weeks. A set of plants regenerated from callus selected on media with various salinity levels (25–100 mM NaCl), with supposable increased tolerance to salt, was obtained. The callus from MS media supplemented with 125 and 150 mM NaCl did not develop somatic embryos.

Testing of somaclones tolerance on salinity

Shoot apices 1 cm in length were cut from plants regenerated from callus tissue selected on MS media with addition of 0–100 mM of NaCl. In this stage of the experiment, according to the method of Pollard and Walker (1990) and Winicov (1994), the explants were placed on MS medium with addition of lower concentration of NaCl – 50 mM, for the assessment of the tolerance level of the examined forms of tomato. 25 explants were placed on each medium (5 explants in one replication). The control consisted of plants not selected in *in vitro* cultures, proliferated on MS medium.

Culture conditions

All the media were supplemented with 8 g · dm^{-3} agar and 30 g · dm^{-3} sucrose, pH was adjusted to 5.7 and autoclaved at 121°C (0.1 MPa) during the time required according to the volume of medium in the vessel. All cultures were incubated in a growth room at a temperature of 24 ± 2°C under 16 h photoperiod from a fluorescent lamp (40 µmol · m^{-2} · s^{-1}).

Statistical analysis

The analysis of variance (ANOVA) was used to calculate the statistical significance and means that differed significantly were determined using Tukey's test at $p < 0.05$. Homogenous groups among the analysed combinations were labelled with successive letters of the alphabet.

RESULTS

In this study, it was found that the plant growth regulators as well as different concentrations of NaCl salt solutions had a significant effect on the induction of callus tissue, proliferation, capacity to form somatic embryos, plant regeneration and the reaction of *S. pimpinellifolium* explants to salt stress.

The effect of plant growth regulators on the callus tissue initiation are shown in Table 1. The highest weight of the initiated callus (0.32 g) was found for explants placed on MS medium supplemented with 2.0 mg · dm^{-3} BAP and 2.0 mg · dm^{-3} IAA. This callus was characterized by dark green colour and loose structure. In turn, the lowest weight of callus (0.06 g) was found for calluses cultured on MS medium without the addition of growth regulators and MS with the addition of 1.0 mg·dm^{-3} BAP. This callus was characterised by light green colour and loose structure (Table 1).

The effect of salinity on callus weight after four weeks cultures are shown in Table 2. The addition to MS medium 25, 50 and 75 mM of NaCl salt had no significant influence on the weight of tomato callus tissue. In contrast, the addition of NaCl to a medium in concentrations ranging from 125 and 150 mM inhibited weight of *S. pimpinellifolium* callus tissue by 64% and 76%, respectively in comparison to the control (Table 2). However, it was observed that explants on MS medium supplemented with 100 mM NaCl increased weight of the developed callus tissue by 21%, in comparison to the control group.

Table 2. Callus weight after four weeks proliferation on MS medium with different NaCl salt concentration
Tabela 2. Masa kalusa po czterech tygodniach namnażania na pożywkach o zróżnicowanej zawartości soli NaCl

NaCl concentration Stężenie soli NaCl [mM]	Callus weight Masa kalusa [g]	Percentage control Procent kontroli
Control – Kontrola	0.89b	100
25	0.70b	79
50	0.73b	82
75	0.69b	78
100	1.08a	121
125	0.32c	36
150	0.27c	24
	$LSD_{\alpha\ 0.05}$ = 0.19 $NIR_{\alpha\ 0,05}$ = 0.19	

Means in the same column followed by the same letter are not significantly different ($\alpha < 0.05$; Least Significant Differences test LSD).
Średnie oznaczone tymi samymi literami nie różnią się istotnie (NIR – najmniejsza istotna różnica; $\alpha < 0,05$).

The concentrations of NaCl used in the experiments had a negative effect on the ability of callus to form somatic embryos (Table 3). In most cases, the number of formed somatic embryos was markedly lower with comparison to that recorded for the control (2.1). It was observed, that callus tissue placed on MS media with addition of 125 and 150 mM NaCl did not formed somatic embryos.

Table 3. Number of somatic embryos on MS medium with different concentration of NaCl salt
Tabela 3. Liczba tworzących się zarodków somatycznych na pożywce MS o zróżnicowanej zawartości soli NaCl

NaCl concentration Stężenie soli NaCl [mM]	No of somatic embryo Liczba zarodków somatycznych	Percentage control Procent kontroli
Control – Kontrola	2.10a	100
25	0.21b	10
50	0.34b	16
75	0.41b	19
100	0.36b	17
125	0.00c	0
150	0.00c	0
	$LSD_{\alpha\ 0.05}$ = 0.20 $NIR_{\alpha\ 0,05}$ = 0.20	

Means in the same column followed by the same letter are not significantly different (α < 0.05; Least Significant Differences test LSD).
Średnie oznaczone tymi samymi literami nie różnią się istotnie (NIR – najmniejsza istotna różnica; α < 0,05).

The obtained populations of *S. pimpinellifolium* samaclones, were in the next stage of experiment tested for salt tolerance on MS media supplemented with 50 mM of NaCl (Table 4).

Table 4. Morphological traits of *S. pimpinellifolium* plant obtained form callus tissue which were selected on MS medium supplemented with 50 mM
Tabela 4. Cechy morfologiczne roślin *S. pimpinellifolium* otrzymanych z kalusa selekcjoowanego na pożywkach MS o różnej zawartości soli NaCl, wyłożonych na pożywkę MS z dodatkiem 50 mM NaCl

NaCl concentration Stężenie soli NaCl [mM]	Percentage of explant developed shots Procent eksplantatów tworzących pędy	Shoot length Wysokość roślin [cm]	No of leaves Liczba liści	Weight of leaves Masa liści [g]
Control – Kontrola	4	1.15b	2.12b	0.62b
25	20	1.15b	3.12b	0.72b
50	20	1.10b	3.62b	0.56b
75	30	0.98b	3.03b	0.78b
100	100	4.23a	7.25a	1.23a
$LSD_{\alpha\ 0.05}$ $NIR_{\alpha\ 0,05}$		0.89	1.23	0.42

Means in the same column followed by the same letter are not significantly different (α < 0.05; Least Significant Differences test LSD).
Średnie oznaczone tymi samymi literami nie różnią się istotnie (NIR – najmniejsza istotna różnica; α < 0,05).
Non of the plants develop root system – Żadna z roślin nie wytworzyła systemu korzeniowego.

The greatest number of explants forming shoots (100%) was found for plants obtained from embryos of callus tissues formed on selection medium containing 100 mM NaCl (Table 4). Populations of somaclones from the other combination of MS media (with addition of 25, 50 or 75 mM NaCl) formed from 20% to 30% shoots. In contrast, only 4% of control

explants forming shoots. Plants regenerated from callus tissue selected on medium with addition of 100 mM NaCl had the longest shoots (4.23 cm), the greatest number of leaves (7.25) and the highest fresh mass (1.23 g). However, somaclones obtained from the other combination of media were at the same level as control in terms of morphological features and constituted a homogenous groups (Table 4).

DISCUSSION

Tomato (*Solanum lycopersicum* L.) is one of the most widely grown, commercially important vegetables throughout the world (Naika et al. 2005). According to numerous authors (Tigchelaar 1986; Kochieva et al. 2002; Rzepka-Plevneš et al. 2004; Zaki and Yokoi 2016), it is due to small range of variability within this species. Wild forms, representative of which is *S. pimpinellifolium*, show greater variability. Studies on obtaining forms resistant to abiotic stresses conducted with the use of traditional methods, are labour-intensive and time--consuming. Therefore, laboratory methods are gaining of popularity as they are more precise, and often cheaper, than conventional methods (Rzepka-Plevneš et al. 2006; Krupa--Małkiewicz et al. 2015; Piwowarczyk et al. 2016). *In vitro* culture has been a useful technique in tomato for the selection of salt tolerant genotypes (Cano et al. 1998; Rzepka--Plevneš et al. 2007; Abdel Latef and Chaoxing 2011; Zaki and Yokoi 2016). Zaki and Yokoi (2016) has been reported that in tomato cultivars *S. lycopersicum* existed a positive correlation between growth of calli and whole plants in saline conditions.

In this study for initiation of callus cultures of wild tomato form, six combinations of MS medium with plant growth regulators (BAP and IAA) were used. The MS medium supplemented with 2.0 mg · dm^{-3} BAP and 2.0 mg · dm^{-3} IAA proved to be superior, and the weight of the callus cultured on this medium was the highest and amounted to 0.32 g. In the next stage of experiment, calli were exposed to different levels of salinity stress *in vitro* ranging from 25 to 150 mM NaCl, and growth was compared to control conditions (no NaCl). Obtained results showed increased tolerance of *S. pimpinellifolium* callus to: 25, 50, 75 and 100 mM NaCl level of salt in the MS medium. Higher concentration of NaCl in MS media (125 and 150 mM) resulted in necrotic lesions and higher rate of necrosis in callus tissues.

The results obtained in this study are in conformity with the study of other authors (Soniya et al. 2001) and de Faria et al. (2002) for *L. pimpinellifolium*, Rzepka-Plevneš et al. (2007) for *L. pennelli* and *L. peruvianum* f. *glandulosum* and Zaki and Yokoi (2016) for cultivated and related wild species of tomato. Cano et al. (1998) obtained callus cultures of *L. penneli* and *L. esculentum* tolerant to 210 mM NaCl. However, the further use of these cultures was significantly limited due to poor regeneration capability of the callus tissue and decreased capacity to form somatic embryos. Differences in terms of tolerance to salinity exhibited by tomato were showed by Cano et al. (1998) and Rzepka-Plevneš et al. (2007) which proves wide variations within one genus. According to mentioned authors *in vitro* screening method could provide an efficient protocol for testing and selecting genotypes for salt tolerance.

In this study, somatic embryos were obtained from fragments of *S. pimpinellifolium* callus tissue selected on media with addition of 0–150 mM NaCl and regenerated on MS media with addition of 2.0 mg · dm^{-3} NAA and 5.0 mg · dm^{-3} BAP. However, the highest number of

developed somatic embryos (2.1) was found for the control medium. The addition of NaCl in concentration: 125 and 150 mM had an inhibitory effect on the capacity to form somatic embryos. Obtained results showed that plants from embryos of callus tested on medium supplemented with 100 mM NaCl, developed the longest shoots. It can therefore be presumed that these somaclones showed higher tolerance to salt stress compared to the other. The trend for wild species to be more salt tolerant is similar to that previously reported by Cano et al. (1998) and Rzepka-Plevneš et al. (2007).

Rzepka-Plevneš et al. (2007) conducted embryogenesis directly from callus tissue considered tolerant to salinity (100 mM NaCl) and reported increased capacity to form somatic embryos in *L. pennelli* and *L. peruvianum* f. *glandulosum* on MS medium containing 3.0 mg · dm^{-3} BAP. Altogether, for the two analysed forms of tomato, they obtained 61 embryos. The studies conducted by Cano et al. (1998) show clear differences in the development of plants *L. pennelli* and *L. esculentum* on MS medium with addition of 70 and 105 mM NaCl. It was found that *L. pennelli* showed higher tolerance to saline stress than *L. esculentum*. According to Domin (2003), most crops are not capable of further development when salinity of the medium exceeds 100 mM NaCl. This is in agreement with the results of own studies, which show that the viability of plants decreased linearly with increasing salinity level. In turn, Zaki and Yokoi (2016) exposed apices and calli of cultivated and wild tomato species to NaCl ranging from 100 to 300 mM. Higher salt tolerance has been reported for wild tomato species than for cultivars of the *S. lycopersicum* in callus culture. This is probably because they have a superior ability to tolerate high levels of Cl^- and Na^+ in their tissues.

CONCLUSIONS

1. The results obtained in this study suggested that shoots and callus culture may be very useful method for rapidly screening and evaluating tomato genotypes.
2. The best medium for initiation of *S. pimpinellifolium* calli was MS supplemented with 2.0 mg · dm^{-3} BAP and 2.0 mg · dm^{-3} IAA. The addition to MS medium 125 and 150 mM NaCl caused necrosis of callus tissue.
3. The population of *S. pimpinellifolium* somaclones obtained from somatic embryos on selective MS media with addition of 100 mM NaCl showed increased tolerance to salinity. Only these explants growing on selective MS media with addition of 50 mM NaCl developed shoots in 100%.

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Abstract. Salinity is a major abiotic stress for plant worldwide which can reduce the average yields of most major crops such as tomato by more than 50%. The response of tomato to salinity is variable depending upon the line or cultivar. The aim of this study was carried out to determine the variation in salt tolerance for wild genotypes of tomato *S. pimpinellifolium*. To initiate callus tissue different combination of plant growth regulators were added to MS medium. The tolerant forms were selected at the callus stage and the stage of plants regenerated through somatic embryogenesis. Callus culture and shoot explants were exposed to different levels of salinity stress ranging from 0 (control) to 25, 50, 75, 100, 125 and 150 mM NaCl. The highest weight of dark green colour callus was observed on MS medium supplemented with 2.0 mg · dm^{-3} BAP and 2.0 mg · dm^{-3} IAA. It was shown that salt stress affected all growth parameters and addition to the MS medium 125 and 150 mM NaCl inhibited callus and somatic embryo initiation. The results obtained in this study suggested that *S. pimpinellifolium* somaclones isolated from callus selected on MS medium supplemented with 100 mM NaCl showed highest tolerance to salt stress.