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THE EFFECT OF *Nicotiana tabacum* L. EXTRACTS ON CULTURES OF TOBACCO CALLUS

Ewa Hanus-Fajerska, Krystyna Ciarkowska
University of Agriculture in Kraków

Abstract. Solanaceae plants are a source of large number of valuable metabolites with multiple use both *in vivo* and *in vitro* conditions. Leaf explants obtained from *N. tabacum* 'Samsun' were cultivated on solidified MS medium supplemented with 3% (w/v) sucrose and enriched with different doses of extracts obtained from seedlings of the same plant cultivar or from *N. tabacum* 'Havana'. The aim of experiments was to verify the effectiveness of such medium additives on callus tissue vitality, proliferation and its organogenetic response. It was ascertained a significant positive influence of all kinds of added extracts on increase of callus fresh weight and the organogenetic capability of tobacco cultures. However, the impact of seedlings devoid of roots *Nicotiana* extracts was significantly lower in both: stimulating of cell proliferation and shoot formation than extracts obtained from whole seedlings. The biological activity was the most effective in the case of genotype compatibility between the plant material from which the extract was derived with the object of biotest.

Key words: plant extracts, tobacco, *in vitro* cultures, callus proliferation, organogenesis

INTRODUCTION

The applications of cultures *in vitro* are innumerable and multiple. Sterile cultures provide an exceptional field for the cooperation between physiologists, biochemists, breeders and other scientists dealing with biotechnology and genetic engineering, one of the major technologies of the twenty-first century [Szopa and Kostyń 2006, Dubert and Płażek 2007]. In some breading protocols, which are connected with biotechnology approaches, it is of prime concern to obtain callus tissue with high regeneration ability. However, the morphogenesis *in vitro* is one of those exceptionally complicated processes, dependent on different internal and external factors. In spite of numerous studies,

Corresponding author – Adres do korespondencji: Ewa Hanus-Fajerska, Department of Botany and Plant Physiology, University of Agriculture in Kraków, ul. 29 Listopada 54, 31-425 Kraków, Poland, tel: (+48) 12 662 52 01, e-mail: ehanus@ogr.ur.krakow.pl; Krystyna Ciarkowska, Department of Soil Science and Soil Protection, University of Agriculture in Kraków, Aleja Mickiewicza 21, 31-120 Kraków, Poland, tel (+48) 12 662 43 70, e-mail: rrciarko@cyf-kr.edu.pl

its biochemical level is still rather poorly understood [Żur et al. 2000, Skrzypek et al. 2007]. On the other hand, by sub-culturing the friable callus pieces in liquid medium with continuous shaking, a suspension of growing cells can be easily obtained. Culture productivity is critical to the practical application of plant tissue culture technology, and until now various strategies have been developed to promote the rate of cell proliferation [Kumar et al. 2004].

Plants are a source of large number of highly valuable compounds. The use of plants to obtain some desired biopharmaceuticals is also possible, but highly effective regeneration protocols are in that case indispensable [Arntzen 1997, Walmsley and Arntzen 2000]. An important role in obtaining the success of *in vitro* propagation plays the optimization of culture medium composition. In this work we focused on the impact of natural extracts on both tissue proliferation and its morphogenetic capacities. The aim of undertaken experiments was to verify the effectiveness of extracts previously obtained from young *Nicotiana tabacum* seedlings on viability and proliferation of callus tissue excised from leaves of the same species, which was treated as model plant. The capability of organogenetic response of short-term callus cultures treated with different doses of extracts added to the culture medium was also verified.

MATERIALS AND METHODS

Extract preparation. Extracts were prepared from fresh green three week-old seedlings of two *Nicotiana tabacum* cultivars, which were obtained by seed germinating on agar solidified medium. In order to prepare extracts whole seedlings or seedlings without roots of *N. tabacum* 'Samsun' and 'Havana' were taken. Plant material was immersed in 50 ml 20% w/v solution of sucrose. Flasks with prepared material were boiled for 3 hours on water bath. The plant material was removed by filtration. To test the biological activity of prepared extracts for 100 ml of MS medium [Murashige and Skoog 1962] – 10 ml (full dose), 5 ml (1/2 dose) and 2.5 ml (1/4 dose) of extracts obtained from whole seedlings of both *Nicotiana* cultivars were added. Extracts obtained from seedlings without roots of both studied cultivars were only added to MS medium in amount of 5 ml. Control treatments constituted the addition of 5 ml of distilled water (C1) or 5 ml of sucrose (C2) to the culture medium respectively.

Experimental scheme. The whole experiment consisted of two phases which lasted ten weeks altogether. In the first one callus cultures were obtained, followed by the second in the course of which shoots were regenerated from callus. Both phases were carried out in Erlenmayer flasks of 100 ml capacity. The experiment was established in 10 replicates and repeated twice. In single experiment a reaction of 600 leaf explants, that is 60 pieces of leaf tissue in each treatment, was evaluated.

The callus proliferation and morphogenic response was compared by determination of the fresh weight, the number and the height of regenerated shoots per tissue clump. The fresh weight of callus was measured after five weeks of tissue proliferation, and the number and the height of shoots regenerated indirectly from callus tissue were calculated after successive period of five weeks.

Callus initiation and shoot regeneration. The object of the biotest was *Nicotiana tabacum* L. 'Samsun'. From greenhouse-grown plants in 3–4 leaves stage, the two upper leaves were taken off. Excised leaves were surface-sterilized by dipping them briefly in 70% v/v ethanol and then for 15 min in 2% v/v sodium hypochlorite solution, followed by three rinses with sterile distilled water. Six leaf explants 5 mm in diameter, were put on 25 ml MS basal medium [Murashige and Skoog 1962], containing 3% (w/v) sucrose and solidified with 0.8% (w/v) Difco agar. Prior to autoclaving at 121°C for 20 min, the pH of the medium was adjusted to 5.8 with KOH. The cultures were incubated in growth chamber at 25ºC ± 2ºC, in darkness for five weeks. Afterwards culture environment was changed. The cultures were maintained at 23ºC ± 2ºC, under continuous/16-h cool-white fluorescent light and 8-h dark period per day. The light intensity was 60 $\mu molm^{-2}$ s^{-1} of Photosynthetic Photon Flux Density. Every two weeks the cultures were transferred to the MS culture medium enriched with respective treatments, and passages were carried out for times. The regenerated shoots above 20 mm high were transferred to the rooting medium consisting of MS macro- and micronutrients diluted thrice and supplemented with 0,25 μM indole-3-acetic acid (IAA). The regenerated plantlets, after potting in sterilized soil, were gradually acclimatized to greenhouse conditions for the next three weeks inside growth chamber at 60% relative humidity in conditions described above. Adopted plants were cultured in the greenhouse.

The results were subjected to unifactor STATISTICA 9, ANOVA analysis and *a posteriori* Fisher's test was used to study a significance of differences between studied objects. Figures were prepared using Excel 2003 program.

RESULTS AND DISSCUSION

Within 7–11 days on explants the callus tissue was initiated, irrespective of experimental treatment put in application. There was no visible differences in callus texture and friability between the treatments. All obtained clump were yellowish, compact and nodular. It was ascertained a significant positive influence of extracts on callus fresh weight estimated after five weeks from the initiation of the culture (tab. 1). Extracts obtained from seedlings without roots of both *Nicotiana* cultivars were less effective in stimulating of cell proliferation than extracts obtained from whole seedlings of both studied cultivars. After addition of 2.5 ml of extracts obtained from 'Samsun' or 'Havana' cultivars as well as 5 ml of sucrose (C2) a measure of callus fresh weight performed after 5 weeks of culture was nearly identical and amounted to 6.46, 6.47 and 6.50 g a flask respectively. The highest callus fresh weight, amounting to above 9 g a flask (tab. 1), determined in treatments to which 10 ml of both extracts, irrespective from the cultivar, per 100 ml of nutrient medium was added.

Table 1 shows that both kinds of added extracts had also a positive impact on the organogenetic capability of tobacco cultures. The biological activity, expressed by a number of regenerated shoots higher than 20 mm, was the most effective in the case of genotype compatibility of the plant material from which the extract derived with the object of biotest that is *Nicotiana tabacum* 'Samsun'. For analogical doses of added

extracts derived from 'Samsun' and 'Havana' differences in number of regenerated shoots were statistically significant. As pointed out in table 1, the highest mean of 8.8 regenerated shoots per single explant was obtained when a full dose of 10 ml of 'Samsun' extract was used. Whereas the level of caulogenesis was significantly lower in the case of the use of extract from 'Samsun' seedlings without roots than from whole plants, amounting to 6.8 and 8.0 shoots respectively.

Table 1. Biometrical parameters of *Nicotiana tabacum* tissue cultured on medium enriched with extracts obtained from *N. tabacum* seedlings

Tabela 1. Cechy biometryczne kalusa *Nicotiana tabacum* kultywowanego na pożywkach wzbogaconych ekstraktami uzyskanymi z siewek *N. tabacum*

Treatments – Traktowanie	FW (g) ± SD	SN ± SD
C1 – 5 ml distilled water – destylowanej wody	$6.38^{a*} \pm 1.02$	$3.0^{a} \pm 1.4$
C2 – 5 ml sucrose – sacharozy	$6.50^{ab} \pm 0.51$	$5.7^{b} \pm 1.0$
1/4 dose – dawki Ex. 1 'Samsun'	$6.47^{ab} \pm 0.63$	$5.5^{b} \pm 1.0$
1/2 dose – dawki Ex. 1 'Samsun'	$9.02^{cd} \pm 0.13$	$8.0^{cd} \pm 0.8$
full dose – pełna dawka Ex. 1 'Samsun'	$9.79^{d} \pm 0.62$	$8.8^{d} \pm 1.7$
1/4 dose – dawki Ex. 1 'Havana'	$6.47^{ab} \pm 0.51$	$3.5^{a} \pm 1.3$
1/2 dose – dawki Ex. 1 'Havana'	$8.80^{c} \pm 0.54$	$6.0^{bc} \pm 1.7$
full dose – dawki Ex. 1 'Havana'	$9.73^{d} \pm 0.42$	$6.8^{bc} \pm 1.3$
1/2 dose – dawki Ex. 2 'Samsun'	$8.37^{c} \pm 0.13$	$6.8^{bc} \pm 1.3$
1/2 dose – dawki Ex. 2 'Havana'	$8.59^{c} \pm 0.44$	$5.60^{b} \pm 1.3$

*the same letters indicate lack of statistically significant differences between mean values at p = 0.05 – jednakowe litery oznaczają brak statystycznie istotnych różnic pomiędzy średnimi dla p = 0,05

FW – mean callus fresh weight per one flask from 6 explants – średnia świeża masa dla 6 eksplantatów

SN – mean number of regenerated shoots (higher than 20 mm) per one explant – średnia liczba pędów (dłuższych niż 20 mm) zregenerowanych z jednego eksplantatu

± SD – standard deviation – odchylenie standardowe

Full dose treatment represents 10 ml of respective extract, Ex. 1 – extract obtained from whole seedlings, Ex. 2 – extracts obtained from seedlings without roots – Pełna dawka stanowi 10 ml odpowiedniego ekstraktu, Ex. 1 – ekstrakt otrzymany z całych siewek, Ex. 2 – ekstrakt otrzymany z siewek bez korzeni

In the figure 1 a–d shares of shoots regenerated from callus tissue cultures cultivated on media supplemented with different kinds and doses of extracts were presented in four ranges of shoot height: below 5 mm, 5–15 mm, 15–25 mm, and above 25 mm. There was observed another pattern of dependences in the case of lower than 5 mm

shoots formation. Independently from the kind and dose of the extract applied the share of shoots lower than 5 mm was the highest, and the share of shoots above 25 mm was the lowest in the total number of shoots regenerated form callus tissue. An addition of both one fourth and a half dose of Ex 1 'Samsun' increased the share of the lowest shoots regenerated in those treatments in a statistically significant way. An addition of one fourth and a half dose of Ex 1 'Havana' increased a number of shoots in the range 5.1–15 mm, and the differences were statistically significant (fig. 1a, b). Whereas, statistically significant impact of the addition of full dose of Ex 1 'Samsun' was observed only in the case of the highest shoots, that is above 25 mm, while the Ex 1 'Havana' caused the formation of the biggest number of the lowest shoots (> 5 mm) (fig. 1 c). A half dose of both 'Samsun' and 'Havana' Ex 2 (without roots) acted similarly as a half dose of both Ex 1 (fig 1d).

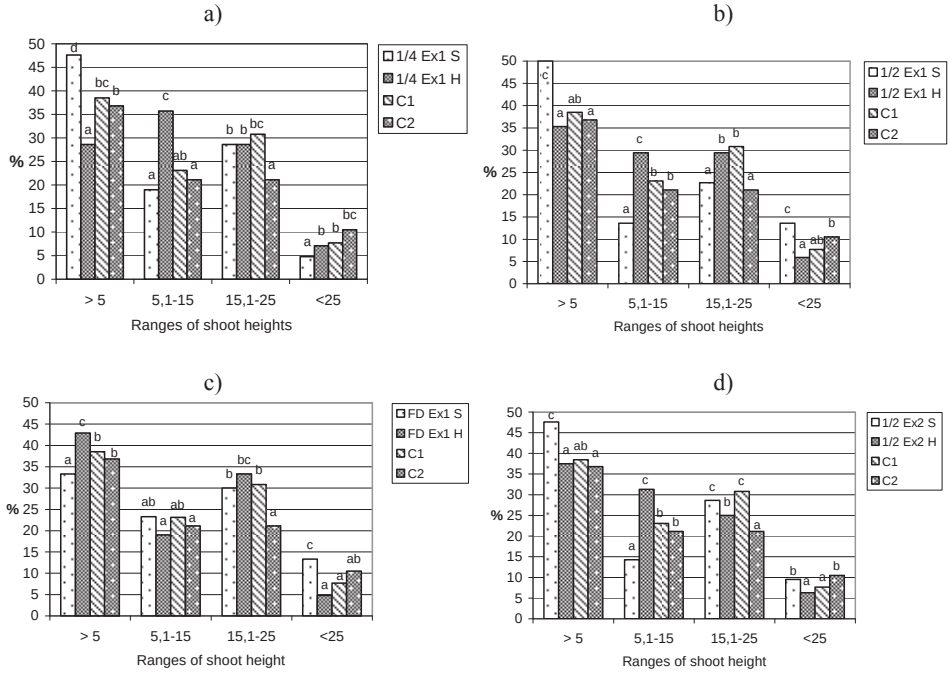

Fig. 1. Share of shoots of given height in the total number of shoots regenerated in *Nicotiana tabacum* 'Samsun' cultures in the respective experimental treatment. Explanations in Table 1.

Ryc. 1. Udział pędów określonej wysokości w całkowitej liczbie pędów zregenerowanych w kulturach *Nicotiana tabacum* 'Samsun' w danym wariancie doświadczenia. Objaśnienia w tabeli 1.

The tobacco industry has largely developed in recent years. However, numerous species belonging to the genus *Nicotiana* are susceptible to numerous pathogens. Resulting diseases, provoke considerable productivity losses of this worldwide grown plant. Moreover the production of disease-resistant material of acceptable quality is

a very complicated task. Additionally, unlike most other annual agricultural crops, tobacco has such small seeds, that they cannot be sown directly in the field. Thus seedlings should be raised in seedbeds or regenerated *in vitro* from tissue of respective cultivar, what ensure that such plant material is protected from pathogens. Tissue and cell cultures are also frequently used in selection of variants with disease-resistance traits in *Nicotiana tabacum* cultivars and breeding lines [Buiatti and Ingram 1991, Gürel 2001]. Because of the excellent response of *Nicotiana* tissues to *Agrobacterium tumefaciens* infection, and relative easy further shoot regeneration, tobacco is continuously used as model plant in numerous biotechnological approaches [Florack et al. 1994, Dieryck et al. 1997, Staub 2000, Gürel 2001, Mungur et al. 2005, Wydro et al. 2006]. Sharp and Doran [2001a, b] even communicated that murine IgG1 can be created in hairy root culture of tobacco.

As in tissue culture protocols an enormous impact is put on medium amelioration, and it is well known that high protein media or an addition of coconut endosperm affect favorably both biomass increase and morphogenesis, it seems not surprising that the tobacco extract addition to experimental culture media exerted beneficial effect. Up to now there have been only few reports on the influence of factors of plant origin that regulate growth and differentiation of tobacco cultures. Organogenesis of tobacco thin-cell-layer explants had been regulated by addition of oligosacharins to the culture medium [Tran Thanh et al. 1985]. Eberhard and coworkers [Eberhard et al. 1989], studying the response of tobacco tissues to the medium enriched with pectic fragments of cell wall polysaccharides, ascertained that oligosacharins from plant cell wall regulate morphogenesis. Afterwards, it has been confirmed that nitrogen form and level influence endogenous level of tobacco plant growth regulators, especially cytokinins [Walch-Liu et al. 2000].

The results of conducted experiments indicate that the highest callus fresh weight could be obtained when relatively high doses of extracts were applied. Moreover, according our results, the higher dose was used, the shorter period of time was needed to obtain rooted plantlets ready to be adopted to *ex vitro* conditions. However, once plants regenerated from tissue cultures were transferred to greenhouse conditions their further growth rate and development was similar, irrespectively of treatment plants came from previously. It is still an open question what molecules with regulatory properties has induced observed reaction of tobacco cultures.

CONCLUSIONS

1. It was ascertained a significant positive influence of *Nicotiana* extracts, obtained from seedlings, added to maintenance medium on callus fresh weight and the organogenetic capability of tobacco cultures.

2. The impact of extracts obtained from seedlings without roots was lower than extracts obtained from whole seedlings in stimulation of callus cell proliferation and caulogenesis

3. The biological activity of extracts was considerably more effective in the case of genotype compatibility between the plant material from which the extract were derived with the object of biotest.

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WPŁYW EKSTRAKTÓW *Nicotiana tabacum* L. NA KULTURY KALUSA TYTONIU

Streszczenie. Rośliny psiankowate stanowią źródło cennych metabolitów o wielorakim zastosowaniu zarówno *in vivo*, jak i *in vitro*. Eksplantaty liściowe izolowane z *N. tabacum* 'Samsun' kultywowano na zestalonej pożywce MS z dodatkiem 3% sacharozy, wzbogaconej zróżnicowanymi dawkami ekstraktów uzyskanych z siewek analogicznej odmiany tytoniu lub z odmiany *N. tabacum* 'Havana'. Celem przeprowadzonych badań była ocena skuteczności wpływu takich ekstraktów na żywotność i proliferację oraz na odpowiedź morfogenetyczną kultur kalusa. Stwierdzono pozytywne działanie zastosowanych ekstraktów zarówno na wzrost świeżej masy kalusa, jak i na potencjał morfogenetyczny kultur tytoniu. Wyciąg uzyskany z siewek pozbawionych korzeni działał mniej skutecznie, zarówno na tempo proliferacji komórek, jak i na kaulogenezę, w porównaniu z otrzymanym z całych siewek. Największa aktywność biologiczna ekstraktów została stwierdzona w przypadku zgodności genotypowej źródła ekstraktu i tkanek zastosowanych jako biotest.

Słowa kluczowe: ekstrakty roślinne, tytoń, kultury *in vitro*, proliferacja kalusa, organogeneza

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