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ORIGINAL RESEARCH PAPER

Changes accompanying proliferative capacity and morphology of *Nicotiana tabacum* L. callus in response to 2,4-D

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Abstract

The common trait of all auxins is a stimulation of cell elongation and also cell division in the presence of cytokinin; both are essential for callus induction and its multiplication. The response of plant tissues to various compounds with auxin activity may be quite different. In this study, the effectiveness of a synthetic auxin, 2,4-dichlorofenoxyacetic acid (2,4-D), instead of the generally applied natural auxin, indole-3-acetic acid (IAA), was tested for the proliferation of *Nicotiana tabacum* callus. The following concentrations of 2,4-D were tested: 0.1, 0.5, 1.0, 1.5, and 2.0 mg dm^{-3}. Callus was derived from stem pith and its proliferation allowed on MS medium through five subcultures at 25°C and in darkness. After each passage, the fresh weight and morphological features of the callus were determined. The 0.5 mg dm^{-3} 2,4-D treatment was the most favorable for producing the greatest increase in fresh weight in each of five subsequent subcultures as well as maintaining normal morphological features for proliferation. However, the 1.0 mg dm^{-3} 2,4-D treatment in comparison with the lowest, 0.1 mg dm^{-3}, was more beneficial when considering regular increases of fresh weight and a better cell cohesion for callus growth.

Keywords

Nicotiana tabacum; 2,4-dichlorophenoxyacetic acid; proliferation; cell divisions; callus morphology

Introduction

Callus may be referred to as a dynamic, histologically heterogeneous system primarily consisting of cells with parenchymatic and meristematic properties. Within this system, vascular formations and meristematic centers differentiate shoot and root primordia [1–3]. Because of the special structure and developmental abilities of callus, it has become a convenient biological material in research on the division and differentiation mechanisms of cells and morphogenesis in plants [4,5]. The capability of callus to form primordia of plant organs such as shoots and roots, and also somatic embryos, enables relatively fast and efficient cloning of many ornamental and vegetable crop species via micropropagation methods [6,7]. An equally important, practical aspect of callus usage is the application of its cells to produce a wide range of primary and secondary metabolites in bioreactors [8,9]. The callus tissue acts as a secondary meristem to revive cell divisions, which are dormant under natural conditions, after scarring or wounds resulting from mechanical damage or the activity of insects or microorganisms [10,11]. In an in vitro environment, callus cells can maintain mitotic activity over a very long period, potentially as long as they can be supplied with optimal requirements for growth, e.g., monthly passages to fresh medium. In some plants, calli possess especially high

vitality and division potential, for example the callus of *Daucus carota* L. Culture of this species was initiated from root tissue with cambium cells in 1939 by Gautheret [12] and Nobecourt [13]; since that initial culture, its growth has continued to this day.

Induction of cell division from primary explants, cell proliferation or regeneration potential depend on various factors, both exogenous and endogenous. One of the most important endogenous factors is the species. *Nicotiana tabacum* L. appears to be a plant with an especially good capability of totipotent expression; it rapidly became a model system for tissue culture [4]. *Nicotiana tabacum* callus can be initiated from small pieces of stem pith by incubation on medium supplemented with indole-3-acetic acid (IAA) and kinetin (KIN), in precise proportions. To maintain the ability of callus cells to display continuous divisions, a higher IAA concentration in relation to KIN is usually necessary. Indole-3-acetic acid is a natural auxin with high physiological activity, however, it suffers inactivation by light and high temperatures [14,15]. For callus induction and proliferation, it is necessary to use a suitable auxin added alone or with cytokinin. Among natural auxins, IAA is often used to stimulate callus proliferation in *N. tabacum* explants. Application of the IAA requires higher concentrations due to its instability in the light and high temperature, whereas the synthetic auxin, 2,4-dichlorophenoxyacetic acid (2,4-D), is unaffected by such conditions. Therefore, in order to obtain the intended morphogenetic effect within a callus culture, it is necessary to apply a suitable higher concentration of this exogenously added auxin. The herbicide 2,4-D is a more effective auxin than IAA in stimulating cell divisions in callus culture [16,17]. The use of 2,4-D results in a lack of light sensitivity and no disintegration by autoclaving temperatures, although it may inhibit organogenesis and induce mutation [18,19].

In this work, we investigated the effects of 2,4-D supplied at different concentrations in combination with a constant concentration of KIN on *N. tabacum* callus cell proliferation and morphological characteristics.

Material and methods

Callus of *Nicotiana tabacum* 'Pereg' was induced within stem pith and then isolated and proliferated on Murashige and Skoog medium (MS) [20] with addition of 3 mg dm^{-3} IAA and 0.2 mg dm^{-3} kinetin. The initial callus was characterized by the nodular structure of its surface, a white color and loose cell cohesion (friable-soft). Callus portions with a volume of about 0.5 cm^3 and mean fresh weight of 120 mg were isolated from the initial callus and maintained in in vitro culture (after the third passage) and subsequently placed on MS medium supplemented with 2,4-D and a standard addition of KIN (0.2 mg dm^{-3}). Five 2,4-D concentrations were applied: 0.1, 0.5, 1.0, 1.5, and 2.0 mg dm^{-3}. After each month, equal sized (0.5 cm^3) callus was cut from the fragments and transferred on to fresh MS medium. A fivefold subculturing of the callus was produced for each concentration of 2,4-D. The media both for the callus induction from primary explants and for further proliferation after pH adjustment in range 5.6–5.8 were solidified with Lab-Agar (Biocorp, Poland) (0.5% w/v) poured to the volume of 15 mL in test tubes and then autoclaved at 121°C for 15 minutes. Callus cultures from the five 2,4-D concentration treatments (each replicated 48 times) were incubated in a plant growth room at 25°C in darkness. After 4 weeks of callus culture before the next subculturing, fresh weights were measured accurately and morphological features (coloration, degree of cell cohesion, and surface structure) were recorded. Morphological features were assessed for 25% of the callus surface or volume in view of their typically heterogeneous morphology.

Data were analyzed by analysis of variance (ANOVA/MANOVA, Statistica 10). For each 2,4-D concentration, 24 calli were used, and the experiment repeated twice. The significances of differences between the means were tested using Duncan's test at a probability level of $p = 0.05$.

Results

The *N. tabacum* callus showed great differences in responses to treatments as evidenced by fresh weight increases and the variability in morphology during subculturing.

Callus subcultured at 0.1 mg dm^{-3} 2,4-D

Comparatively high callus biomass increase was achieved at 0.1 mg dm^{-3} 2,4-D, and after all passages, mean fresh weight amounted to 173 mg. During the first three passages, greater differences of fresh weight increase were found in comparison to later passages: 16, 20, and 15 mg, respectively. By contrast, during the last two passages these differences were smaller, generally between 6 and 12 mg (Fig. 1a). The callus color was grey and cream in 50% of calli during the first two passages. In the next three subcultures, the cream color increased and accounted for 75% of calli (Fig. 2). These calli were characterized by high cell cohesion (100% compact calli) and a nodular surface (Fig. 3–Fig. 5a). Beyond the first passage, a few root primordia were recorded, but they were observed in 75% of calli, and their presence was again noted in all the next passages. Root organogenesis was only initiated at a concentration of 0.1 mg dm^{-3} 2,4-D.

Callus subcultured at 0.5 mg dm^{-3} 2,4-D

For the 0.5 mg dm^{-3} concentration of 2,4-D, calli were characterized by the highest value of fresh weight increase, on average 187 mg from all five passages. However, the increases in fresh weight in subsequent subcultures ranged from 7 to 31 mg. The fresh weights achieved ranged widely, from 141 mg after the first passage to 226 mg after the fifth (Fig. 1b). The callus color was more varied than in the previous 2,4-D treatment. Calli were cream or grey colored (the latter, 50–70%) during the majority of passages (Fig. 2). However, after the third passage, calli appeared with light-brown sectors occupying <25% of the surface; for this reason, it is not included in Fig. 2. The analysis of the cell cohesion indicated that calli were mostly fragile (75%) with a lesser proportion (25%) possessing large cell cohesion through the first three passages (Fig. 5b). However, after the fourth and fifth passage all calli were fragile (Fig. 3). The most homogenous morphological trait of calli was the structure of the surface, which was nodules during five successive passages (Fig. 4). For this 2,4-D concentration variant, organogenesis did not occur at a later stage.

Callus subcultured at 1.0 mg dm^{-3} 2,4-D

Doubling the 2,4-D concentration in comparison to the above brought about a reduction in the mean increase of fresh weight for all passages, significantly lower, and amounting to 148 mg. The differences in fresh weight increases between individual subcultures were also small: 5, 12, 8, and 8 mg, respectively (Fig. 1c). From the first passage, all incubated calli assumed a uniform, grey color over their entire surface, and the color remained in the subsequent passages (Fig. 2). Taking into consideration the degree of cell cohesion that increased in the 0.5 mg dm^{-3} 2,4-D treatment, all the calli were soft until the fifth passage (Fig. 3). Other callus morphological features, notably the nodular surface structure, were observed in all calli from the first to the last passages (Fig. 4, Fig. 5c).

Callus subcultured at 1.5 and 2.0 mg dm^{-3} 2,4-D

Calli proliferating on the medium at the two highest 2,4-D concentrations were similar with regard to their effectiveness in fresh weight increase and gave means of 119 mg and 110 mg, respectively, after all five passages (Fig. 1d,e). At both concentrations, the small increase of fresh weight was observed after each subsequent passage. However, at the highest 2,4-D concentration, the differences among callus biomasses were

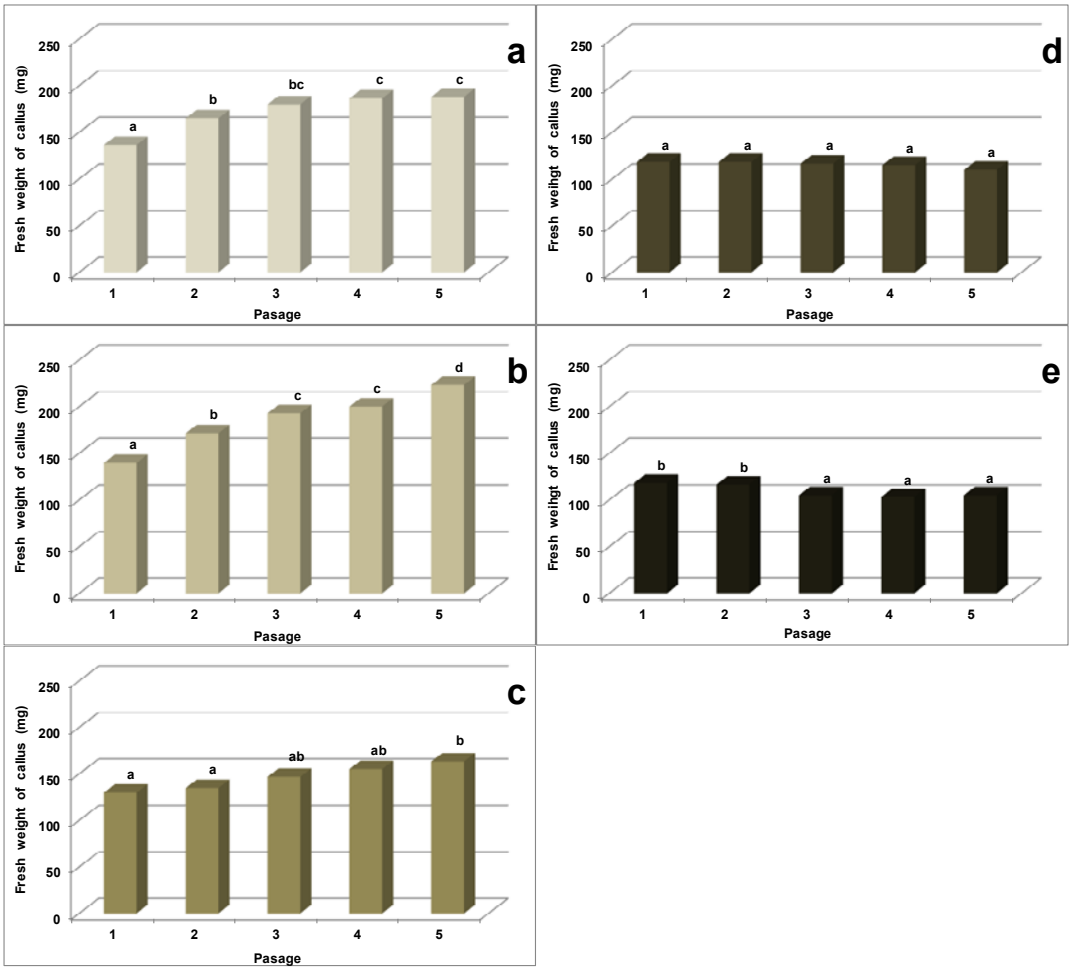

Fig. 1 Proliferation of *Nicotiana tabacum* callus on MS medium with various concentrations of 2,4-D: 0.1 (**a**), 0.5 (**b**), 1.0 (**c**), 1.5 (**d**), and 2.0 (**e**) mg dm^{-3} during five subcultures. Values followed by the same letter are not significantly different at $\alpha = 0.05$ according to Duncan's test.

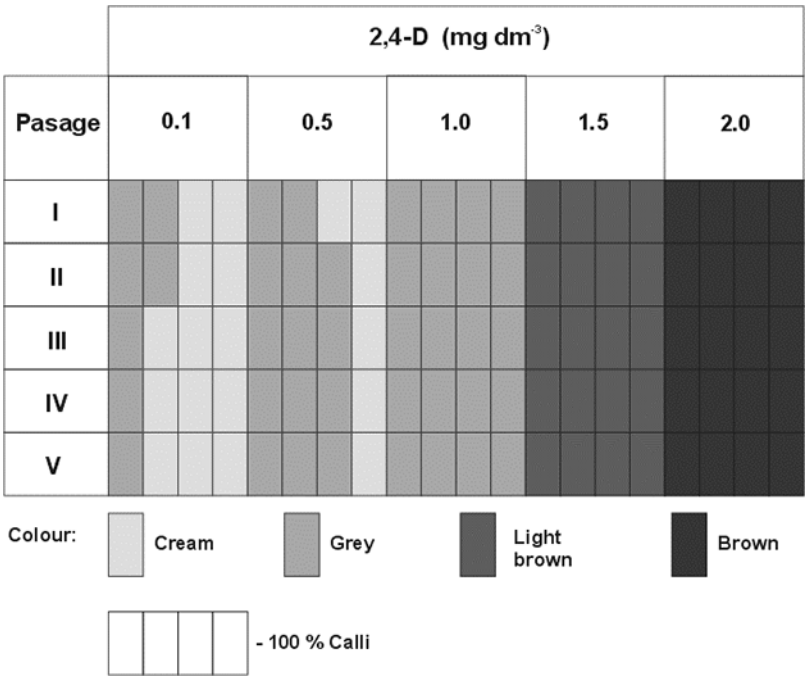

Fig. 2 Morphology – coloration of the *Nicotiana tabacum* callus during its proliferation on MS medium with individual 2,4-D concentrations in successive five subcultures.

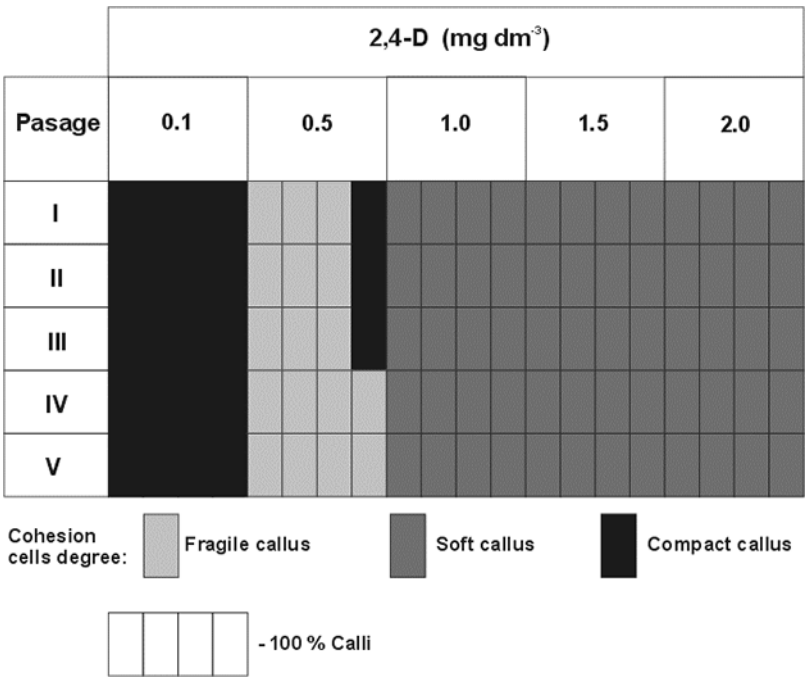

Fig. 3 Morphology – cell cohesion degree of the *Nicotiana tabacum* callus during its proliferation on MS medium with individual 2,4-D concentrations in successive five subcultures.

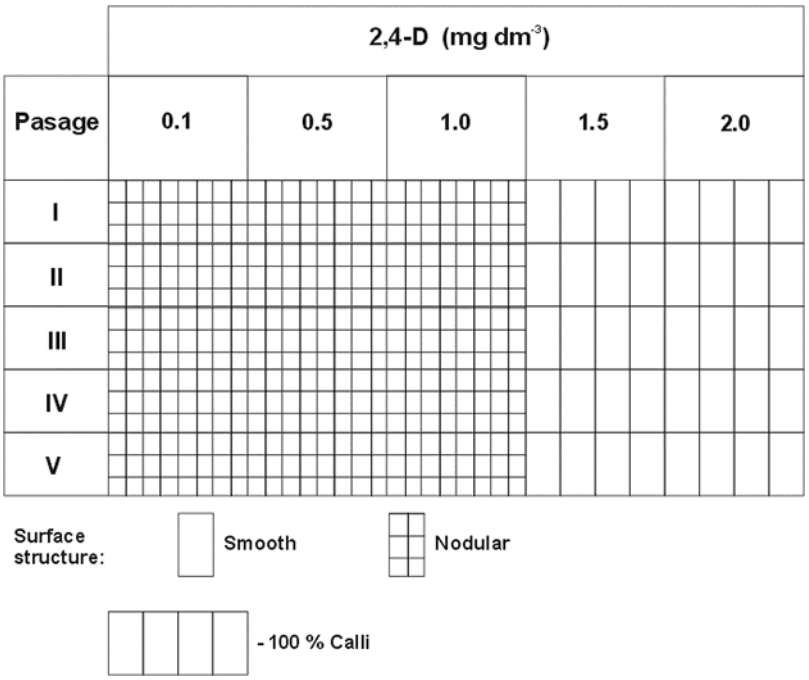

Fig. 4 Morphology – surface structure of the *Nicotiana tabacum* callus during its proliferation on MS medium with individual 2,4-D concentrations in successive five subcultures.

slightly bigger after each passage. The common response to both concentrations was an alteration in callus morphology. Brown coloration, not noted in any of the lower concentrations, was noted and was maintained through all passages. However, at the 1.5 mg dm^{-3} 2,4-D concentration the pigmentation assumed a brighter hue (Fig. 2, Fig. 5d,e). Another different feature of the callus morphology here was the surface structure, which was not nodular but smooth (Fig. 4). However, there was a similarity in the third morphological feature considered, the degree of cell cohesion, which was midrange, resulting in a soft callus (Fig. 3).

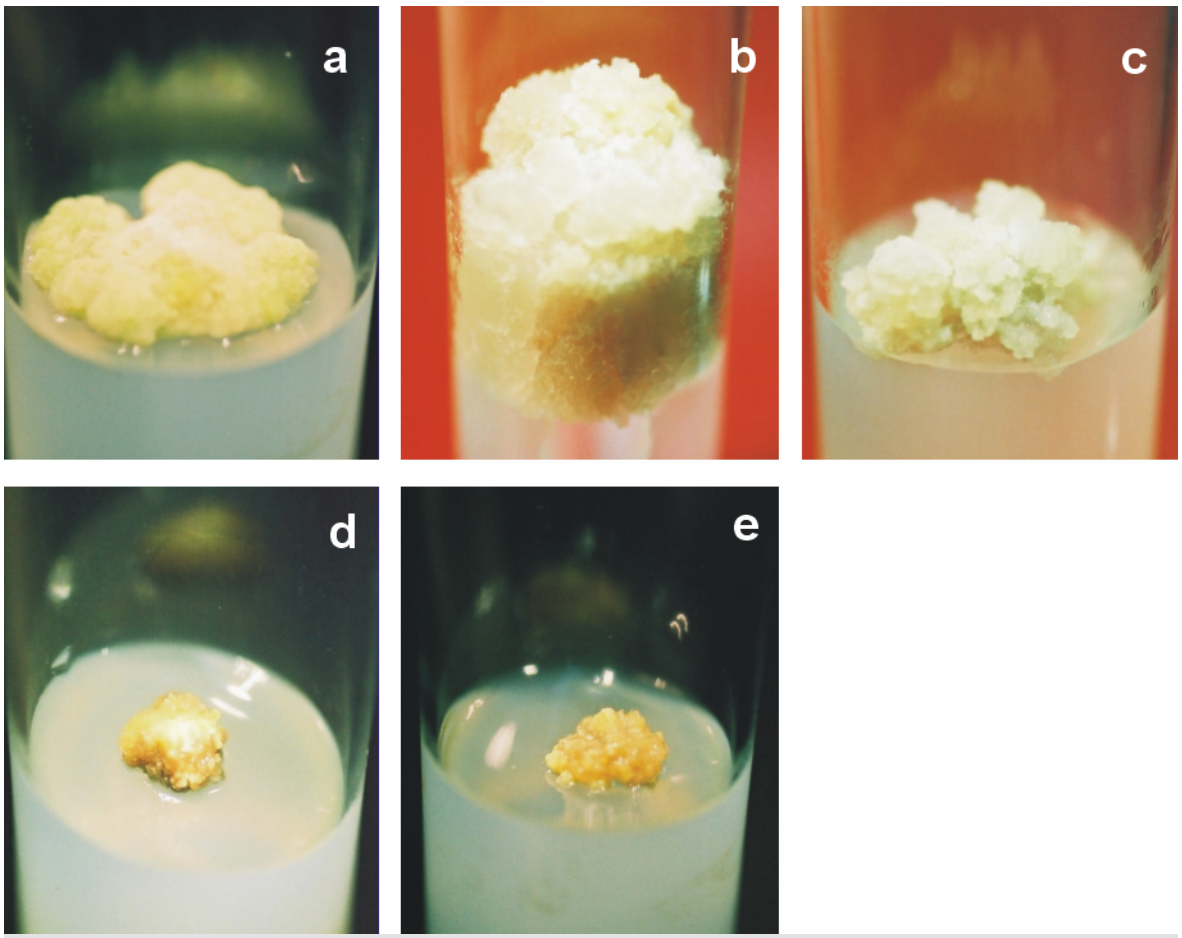

Fig. 5 Calli cultured on MS medium supplemented with kinetin (0.2 mg dm^{-3}) and 2,4-D at concentration: 0.1 (**a**), 0.5 (**b**), 1.0 (**c**), 1.5 (**d**), and 2.0 (**e**) mg dm^{-3} after the fifth passage.

Discussion

In order to induce callus and stimulate its proliferation, it is necessary to use a combination of auxin with cytokinin. A suitable balance of these plant growth regulators will potentially give the intended result, callus tissue formation. However, such a tissue does not usually have a homogenous structure. Callus morphology as well cell proliferation ability are greatly dependent on the type and concentration of the applied auxin. In the present study, formed *N. tabacum* callus was characterized by various morphologies and the growth rate was affected by the concentration of 2,4-D.

At the lowest applied 2,4-D concentration in combination with kinetin, the auxin effect was manifested by a comparatively high stimulation of increase in the fresh weight despite the dominant effect of kinetin. A similar effect on the efficiency of callus induction at relatively low 2,4-D concentrations in leaf segments of *N. tabacum* was also observed [21]. At almost an equal ratio of this auxin to kinetin (2.5:2.0 mg dm^{-3}), compact callus formation was achieved with a 90% frequency and one of the highest increases in fresh weight was observed. However, 2,4-D at the same concentration (2.5 mg dm^{-3}) with another auxin – naphthalene acetic acid (NAA) but in ratio 1:15, stimulated a similar quantity of friable callus. Naphthalene acetic acid without 2,4-D in the medium gave the smallest increase in callus biomass. The data presented here also support the high level of activity of 2,4-D. It seems that the uptake and availability of this auxin in plant tissues may be important for its activity. As Viana et al. [22] reported, the high physiological activity of 2,4-D within *N. tabacum* callus in comparison with *Dioscorea* sp. (a monocot) callus results from the fact that the major products of 2,4-D metabolism in the former one are sugar esters. As a result of the stability of these compounds in tissue, their hydrolysis and the release of free 2,4-D from the conjugated form are possible at any time for giving physiological activity. This suggests that 2,4-D is a very effective auxin that may have influence on the DNA expression. Yamada et al. [23] were the

first, who reported that exogenously incorporated 2,4-D can form 2,4-D/lysine-rich histone complexes and impact on the decrease of the histone proteins and simultaneous increase of nonhistone proteins during callus induction. In turn, the kinetin effect was revealed by the callus morphology, especially when considering the degree of cell cohesion giving a compact callus in many cases, so characteristic of cytokinin dominance over auxin in the medium [24]. Distinct cell cohesion of the callus in the presence of kinetin, as suggested Kirkham and Holder [25], may result from its impact on cell wall hardening, and thus causing an increase in cell water potential. Furthermore, this change in potential means that cells became less liable to take up water externally. Kinetin is located in nuclear DNA where it is formed as a product of oxidative modifications of DNA [26]. Moreover, this cytokinin protects DNA from peroxide-induced formation of 8-oxodG in a Fenton reaction. The effects of kinetin on cell metabolism are observed on transcriptional, translational, and post-translational levels [27].

The presence of root primordia, admittedly rare but occurring from the first to last subcultures, demonstrates the advantage of the 2,4-D on the differentiation process. In this study, it is very interesting that in *N. tabacum* root organogenesis takes place at the significantly higher concentration ratio of IAA:KIN [28]. It could point to the very strong stimulation of root induction by 2,4-D. This synthetic auxin may have an active effect on the derepression of ARF (auxin response factor) proteins, which can be activators or repressors of transcription genes in the auxin pathway. The modules of auxin signaling play both a key role in root organogenesis and also callus formation [29]. Except for the rhizogenesis, the increase of fresh weight of calli was observed in the present study.

Over 2 times higher a concentration of 2,4-D to that of kinetin appeared to be the most effective for callus proliferation, which was reflected by the highest increase in fresh weight and the rate of this increase. This response may confirm the major influence of this auxin on cell proliferation even at a relatively low concentration. The growth of callus is a result of cells division and elongation. Auxins possess a specific property, due to the fact that binding to their receptor, AUXIN BINDING PROTEIN 1 (ABP1), they trigger signal transduction which may result in cell elongation as rapidly as within a few minutes [30,31]. It is probably involved with proton pump ATPase activity causing H^+ extrusion and also extracellular acidification, expansion activity, wall loosening, uptake of K^+, and water uptake to sustain cell expansion [32]. The auxin receptor ABP1 recognizes 2,4-D as IAA even though it is not as good a substrate as IAA. However, IAA may be inactivated relatively quickly but 2,4-D can remain in the cell much longer [33]. In the case of the callus studied, most growth occurred at the relatively low 2,4-D concentrations in comparison with other plant species such as *Achyranthes aspera* L. [34], where a similar effect was achieved at 4 times higher a concentration, and in *Solanum tuberosum* L. at even 6 times higher [35]. The fact that the advantageous effect on proliferation was achieved at 0.1 mg dm^{-3} 2,4-D concentration confirms its effective induction of cell divisions that can occur even in the absence of cytokinin in the medium [36]. In our study, there was a strong effect of 2,4-D on the morphology of calli and especially on the cell cohesion, which decreased in comparison with the lower 2,4-D concentrations and gave a friable callus. The rapidly proliferating calli are characterized by friable internal structure [3]. However, in the case of the 1.0 mg dm^{-3} 2,4-D treatment, increases in the fresh weight occurred in subsequent subcultures and they had lower values of fresh weight in comparison with the lower 2,4-D concentration, but they were more regular, which could point to some stabilization in the callus growth rate. This conclusion could be supported by the fact that calli incubated at this concentration transformed from fragile to soft and then the cell cohesion increased marginally. The cell cohesion was characteristic for a callus with stable growth [37]. Moreover, the grey pigmentation present for all calli at 1.0 mg dm^{-3} 2,4-D concentration may result from distinct cell hydration that is one of the characteristic effects of auxin action [38]; this is favorable for callus proliferation [39].

Browning at different intensities within calli was characteristic of the two highest 2,4-D concentration treatments. The appearance of such a callus color is related to the presence of phenols, which often accompany the tissue cultures, especially in woody plants. The oxidation of phenols by polyphenol oxidase (EC 1.10.3.1-2) or peroxidase (EC 1.11.1.7) leads to the formation of phenolic bridges between cell wall components (polysaccharide and lignins, polysaccharide and structural proteins) [40]. The formation

of these bridges follows a loss of extensibility of the cell wall and consequently the restriction of cell growth. It could be seen in this study as a decrease in the fresh weight of brown calli during successive subcultures. Similarly, with the browning of callus in *Pinus sylvestris* L., growth rate was low particularly between the second and the third culture weeks, when the increase of peroxidase activity is also at its highest [41]. In the herb *Boerhavia diffusa* L., it was observed that while the callus browning was intensified, the simultaneous increase in peroxidase activity was a result of the decrease of the dividing cells in proportion to nonmeristematic elongated cells in the callus [42]. Also in the highest 2,4-D concentration treatments (3 and 4 mg dm^{-3}) in *Chrysanthemum* sp., a brown color of the calli resulted from the production of secondary metabolites in the form of phenolic, terpenoid, isoprenoid, and flavonoid groups [43]. Moreover, 2,4-D may induce the production of ethylene [15], which promotes oxidative and nitrosative stress causing disturbances in the actin cytoskeleton involved in post-translational modification of actin by oxidation and nitrosylation [44]. This may then cause an alternation of peroxisomes and mitochondria mobility. It is known that the peroxisomes are the main antioxidant organelles in the cell. Therefore, the ROS-removing function of the peroxisomes may be disturbed. Like the other auxins, 2,4-D may also activate genes of ABA biosynthesis that together with ethylene genes initiate ROS overproduction [15,45]. The above-mentioned action of 2,4-D may cause a slowing or an inhibition of cell growth, which was observed in the present study.

Conclusions

- The greatest increase in the fresh weight of the callus was observed at a 2,4-D concentration of 0.5 mg dm^{-3}. Callus incubated in this treatment possessed a beneficial friable texture that was maintained through all passages, as well as a grey and cream coloration pointing indirectly to proper cell hydration. The above features are advantageous for cell proliferation and thereby they give an opportunity for long-lasting callus culture. Calli of *N. tabacum* growing at the lowest 2,4-D concentration were characterized by a very high fresh weight increase. However, this concentration treatment is not suitable for callus multiplication when considering the simultaneous formation of root primordia through all passages and also a very strong cell cohesion (compact callus).
- In the case of callus incubated on 1.0 mg dm^{-3} 2,4-D, compared with the foregoing concentration, smaller increases in fresh weight were achieved. However, in view of the advantageous cell hydration and cell cohesion, this concentration variant may be potentially considered for callus cell proliferation.
- Both the weak growth and morphological features of calli in the 1.5 and 2 mg dm^{-3} 2,4-D concentration treatments may suggest the occurrence of a very early cell differentiation or cell necrosis within calli. This concentration range is therefore not useful for intensive callus formation.

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Zmiany związane ze zdolnością do proliferacji i morfologią kalusa *Nicotiana tabacum* L, w odpowiedzi na różne stężenia 2,4-D

Streszczenie

Do indukcji i namnażania kalusa niezbędne jest zastosowanie odpowiedniej auksyny, podanej samodzielnie lub z cytokininą. Spośród naturalnych auksyn IAA jest często wykorzystywana do stymulowania proliferacji kalusa *Nicotiana tabacum*. Zastosowanie IAA wymaga wyższych stężeń ze względu na nietrwałość, wynikającą z wrażliwości na światło i wysoką temperaturę. Takich ograniczeń nie posiada syntetyczna auksyna 2,4-D. Celem tej pracy była ocena przydatności 2,4-D do proliferacji kalusa *Nicotiana tabacum* z następującymi wariantami stężeń 0.1, 0.5, 1.0, 1.5 i 2 mg dm^{-3}. Proliferację kalusa z rdzenia łodygi przeprowadzono na pożywce MS przez 5 pasaży bez dostępu światła i w temperaturze 25°C. Po każdym pasażu określono świeżą masę i cechy morfologiczne kalusa takie jak barwa, struktura powierzchni i stopień spójności komórek.
Spośród badanych stężeń 2,4-D, najefektywniej stymulującym było 0.5 mg dm^{-3} ze względu na najwyższy przyrost świeżej masy w każdym z pięciu kolejnych pasaży oraz sprzyjające proliferacji cechy morfologiczne kalusa. Wariant stężenia 1.0 mg dm^{-3} 2,4-D w porównaniu z 0.1 mg dm^{-3} okazał się korzystniejszy, ponieważ charakteryzował się regularnymi przyrostami i lepszą dla namnażania spójnością komórek kalusa.

