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Synthetic auxin herbicide 2,4-D and its influence on a model BY-2 suspension

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Abstract
2,4-D is a broadly used auxin herbicide. The presence of the 2,4-D synthetic auxin in the medium is imperative for long-term BY-2 tobacco suspension viability. The precise mechanism of this symbiosis of the suspension and the synthetic auxin remains unclear. Our goal was to study the hormonal regulation of the growth of the cell suspension; and to describe the experiments clarifying the interaction between the chosen growth regulators and phytohormones on the cellular level, specifically between the 2,4-D synthetic auxin and the native stress phytohormone — ethylene. This study examined the influence of low 2,4-D concentrations stimulating cell growth *in vitro* as well as the influence of high herbicide concentrations on the model tobacco BY-2 suspension. The culture took 6 days. Different parameters were evaluated, including the influence of different 2,4-D concentrations on the production of the phytohormone ethylene and its precursor 1-Aminocyclopropane-1-carboxylic acid (ACC) in the tobacco cells. The content of 2,4-D in the cells and the medium was established. The observations of the morphological changes showed that a heavy impregnation of the cell walls taking place depending on the concentration of 2,4-D. A dramatic increase in protective polysaccharides and a remodulation of the cell walls by the formation of a pectin shield in artificial conditions were expected and observed. At the same time, massive production of the stress phytohormone ethylene took place, and, because of that, plant mutagenicity, anomalous tumour-type proliferation growth, and the production of supercells were observed. The hypothesis of the protective shield is discussed.

Keywords: BY-2 cell suspension; 2,4-dichlorfenoxyacetic acid; 2,4-D; ethylene; plant mutagenicity; pectin shield

1. Introduction

Bright yellow-2 (BY-2) is a derived cell suspension model culture from the callus induced from tobacco sprouts. The plant is one of the few model systems steadily forming one-row multicellular chains. BY-2 has qualities that are advantageous and necessary to study biological processes in the cell and is hence used very broadly [1,2]. These traits, however, don’t occur without an auxin-character synthetic substance, such as 2,4-Dichlorophenoxyacetic acid (2,4-D), present. The acid allows the suspension to survive for months and proliferate. It is impossible to replace the high growth effect of 2,4-D in a liquid medium with any other natural, or synthetic, auxin. Due to its advantageous qualities, it is often used to study the phytohormones’ influence on plant cells, secondary metabolism

processes, as well as programmed cell death. Currently, it is also used in biotechnological practice for cell-free protein production [3,4,5]. 2,4-D has long been used as a herbicide for dicotyledons, even though the substance counts among dangerous environmental contaminants. Its herbicide effect lies in overdosing the plants with auxin, which disrupts the equilibrium and causes apoptosis [6,7].
The danger of 2,4-D when combined with other substances is proven by cases of mutations in humans, who are born with birth defects even after 50 years after its use during the Vietnam War. Four million Vietnamese have been fighting its mutagenic effects ever since 1961 when the American armystarted contaminating the Vietnamese jungle as well as the agricultural land with a combinationof 2,4-D and 2,4,5-T, used under a code mark 'Agent Orange' to destroy the vegetation to get easier passage for the American forces. [8] Ethylene is the only natural gaseous phytohormone with numerous physiological effects. In its physiological concentration, its effects are very ambiguous. Therefore, it cannotbe considered nor inhibitor or stimulator; but rather a growth modulator. Ethylene can stimulate and inhibit root and stem growth and induce the formation of root hairs and flower and fruit ripening. It can contribute to senescence and abscission of flower petals or plant leaves. Furthermore, it can regulate the reaction to biotic and abiotic stress. It is impossible to predict the plant tissue reaction to the exogenic ethylene application with certainty.
Ethylene is commonly used for agricultural purposes and directly influences the health of the plants as they adapt to changes in conditions and reproduce. One of the first reactions of the plants to the provoked stress is to increase ethylene production. This is why ethylene is often called 'stress phytohormone'. It is the most well-known signalling molecule participating in the plant defence response. Its physiological significance in stress conditions is not well understood, and it is a possible side-effect of the plant's response to exposure to stressors [9,10].

Kaźmierczak [11] researched how the cytoskeleton influences the regulation of induced cell death in tobacco BY-2 cells.The study shows that programmed cell death (PCD) in response to kinetin correlates with an arrest of the cell cycle, deregulation of DNA replication, a loss of plasma membrane integrity, followed by the nuclear envelope permeabilisation, a cytosolic calcium increase, a callose deposition increase and the loss of microtubule and actin integrity. In the tests, kinetin, mediated by calcium, led to the breakdown of the cytoskeleton and, subsequently, resulted in PCD. Zheng [12] identified Long noncoding RNAs (lncRNAs) in BY-2 cells, studied their response to methyl jasmonate (MeJA), and investigated their potential defence against stimuli, using Nicotiana tabacum. They analysed the cis-regulated lncRNAs and their target genes and discovered that some are responsible for different biological functions and metabolic patterns and might have an impact on the plant's defence and stress resistance. They found out that MeJA-responsive lncRNA target genes may help nicotine synthesis and disease and insect resistance, by regulating the target genes in BY-2 cells. Salcedo [13] investigated the use of bentonite (Bent) to adsorb 2,4-dichloro phenoxy acetic acid (2,4-D) pesticide to avert the risks of growth retardation senescence, and cell death in non-target plant species endangered due to soil and water contamination. Arabidopsis thaliana was used for the analysis of the 2,4-D adsorption by Bent-DDA, and lettuce to research phytotoxicity prevention. 2,4-D significantly inhibited the root growth of Arabidopsis seedlings. When pre-treated with Bent-DDA, the seedlings demonstrated a root growth similar to 2,4-D non-treated seedlings. Lettuce plants were used to examine potential phytotoxicity prevention by Bent-DDA. When pre-treated with Bent-DDA, the plants showed reduced sensitivity to 2,4-D including chlorophyll content and biomass increment in comparison with non-treated plants. Moreira [14] assessed the environmental implications of the use of 2,4-D herbicide and fipronil insecticide (alone and in combination) on the native

tropical chironomid Chironomus sancticaroli. The risk lies in the exposure of benthic organisms to pesticides and insecticides through contaminated water and sediments, which potentially result in food web damage. Toxicity tests were run on commercial products containing fipronil (Regent® 800WG) and 2,4-D (DMA® 806BR) to examine the products' effects on larval survival, growth (body length and biomass), head capsule width, development, and mentum deformities. Fipronil decreased larval survival and increased the incidence of deformities with increasing concentrations. The highest test concentration of 2,4-D decreased the head capsule width but didn't affect the larval development at any of the evaluated concentrations. The results showed that fipronil represents an environmental risk for C. sancticaroli populations and has synergistic effects in combination with the herbicide 2,4-D. Chan [15] analysed the behaviour of a cell polarity protein BASL (Breaking of Asymmetry in the Stomatal Lineage) of cultured tobacco BY-2 cell filaments and in protoplasts to examine through what mechanisms the cell polarity is established and how it is coupled to growth. That is important as the tissue cell polarity can influence the cells' growth and division. The study shows that ectopic BASL becomes polarly localised in tobacco BY-2 and is found at the developing tips of cell filaments. The polarity can change over the course of the cell cycle and cannot be treated with microtubule, actin or auxin transport inhibitors. The results suggest that plant cells have an intrinsic ability to polarise and can direct this polarity to cause anisotropic growth. Tichati [16] examined the hepatoprotective effects of selenium on toxicity induced by 2,4-dichloro phenoxy acetic acid in Wistar rats. The rats were treated with selenium and 2,4-D with a control group present. An increase in liver function markers and a decrease in glutathione (GSH) content revealed the hepatotoxic and pro-oxidant effects of 2,4-D application. The microscopic observation and histology of the 2,4-D-treated rats' liver showed lesions causing perivascular inflammatory infiltration around the vessel, sinusoidal dilatation and vacuolization of hepatocytes. Selenium treatment led to a reduction in toxicity and could potentially prevent the hepatotoxicity of the 2,4-D pesticide. Rukmana [17] employed a near-infrared femtosecond (fs) laser to introduce megadalton molecules into a cytoplasm of an intact single plant cell. The fs laser has a remarkably precise perforation capability, but has, until now, only been used for mammalian cells. The gene introduction to plant cells is vital for the understanding of individual gene functions and the engineering of plant functions such as high-yielding ability and disease and stress resistance. The intense fs laser pulses perforated the cell wall and membrane of Tobacco BY-2, and 2 MDa dextran molecules were introduced through the pore. Less thermal damage was observed and the enzyme treatment enhanced the diffusion of large molecules through the cell wall. Bernat [18] investigated the effect of the ascomycetous fungus Trichoderma harzianum on wheat seedlings (Triticum aestivum L.) treated with 2,4-dichloro phenoxy acetic acid (2,4-D). The herbicide is commonly used in wheat cultivation, but can seriously affect its growth. The seedlings exposed to the herbicide showed increased lipid peroxidation, growth inhibition and elevated levels of oxylipins. Contrarily, Trichoderma harzianum treatment proved stimulating to the seedlings' growth. The wheat seedlings treated with 2,4-D and Trichoderma harzianum showed a similar level of lipid peroxidation to that in the control group and no increase was seen in oxylipins and phospholipase D activity. T. harzianum could potentially help mitigate the toxic effect of 2,4-D on wheat seedlings. Olmos [19] examined the changes provoked by salt adaptation in tobacco BY-2 cell cultures to arabinogalactan proteins (AGPs) distribution and contents. The AGPs influence cell differentiation and expansion, tissue development and somatic embryogenesis, and they also affect abiotic stress responses. The AGPs were observed to concentrate in the culture medium of the salt-adapted tobacco cells. The study shows that the salt adaptation resulted in a significant reduction of the cytoplasm, plasma membrane and tonoplast content of epitopes. The application of the Yariv reagent led to cell death in control cells but not in salt-adapted tobacco cell cultures. Conclusively, the

AGPs could potentially carry sodium through vesicle trafficking to the vacuoles in salt-adapted tobacco BY-2 cells and this mechanism could contribute to sodium homeostasis during salt adaptation.

Studies have shown some plant species respond to the increased contamination by heavy metals in the environment through polysaccharides production increase, including pectin production, as seen in the studies by Colzi [20] and Wang [21]. An important trait of pectin substances is the ability to bind divalent and trivalent heavy metals, as explained in the study by Mehees-Smith [22]. Pectin substances in the cell wall compensate for heavy metals and disable their reception into the cytoplasm, which in turn increases the plant's resistance to heavy metals in the environment. The ethylene stress phytohormone production seen in this study supports the pectin hypothesis. This hypothesis is based on the production of protective substances in the cell walls, that is, the thickening of the walls depending on the concentration of 2,4-D in the growth medium, and therefore also the newly proposed theory of uncontrolled cell division (plant cancer). Plant cancer is known in nature. Among others, *Agrobacterium tumefaciens* bacteria induces plant tumours, as shown in the study by Torres [23]. Until now, the cell proliferation of plant suspension cultures has been considered a genetically regulated cell division. This work represents a fundamental change in the attitude to this issue. Tumour cells have a different – a modified – DNA to normal cells. The produced ethylene activates the pectin esterase enzymes that split the cell chains. The highest amount of ethylene is produced at low 2,4-D concentrations, the cell chains quickly collapse, and the cells significantly grow in volume. At higher concentrations, the production decreases, the cells stay organised in chains, and the cell walls thicken abnormally. The protective pectin substances and hemicelluloses should be determined quantitatively, which is why this study represents a hypothesis.

Until now, low 2,4-D concentrations used for *in vitro* cultivation have never been considered a potential stressor. Low 2,4-D concentrations are currently used to stimulate the division growth of the plant tissue culture in *in vitro* conditions. Higher concentrations, however, have a herbicidal effect *in vivo*. The study aimed to examine the hormonal and trophic regulation of the cell suspension growth and describe the experiments performed to determine the significance of the chosen groups of growth regulators and phytohormones (2,4-D and ethylene). Derived from the mesophyll of the leaves of the tobacco variety "Bright Yellow", the BY-2 tobacco cell suspension was chosen for the experiment. The cell suspension consists of cell chains with a generation time of 7–9 days, depending on the culture conditions. It requires the 2,4-D synthetic auxin in the medium, as it is otherwise incapable of growth. Until now, the BY-2 suspension dependence on the 2,4-D in the cultivation medium has not been explained, which is what makes this study unique and original. The findings from the suspension growth, 2,4-D content and ethylene production will clarify the interaction between the studied phytohormones and the cellular-level growth.

2. Experimental setup and material

2.1. Plant material and experimental varieties

The cell suspension culture (*Nicotiana tabacum* L.) of the tobacco BY-2 line was used to perform the experiments as per the study by Nagata [24]. Regular passaging into fresh growth medium was necessary in order to maintain the suspension viability. The subcultivation (suspension to a new liquid medium in ratio 1:9) was always performed after seven days into 100 ml Erlenmeyer flasks and the liquid-modified LS medium (Linsmaier and Skoog [25]), fortified with 30 g/l saccharose and 1μM of 2,4-D with a pH of 5.7. The cultivation was performed on the INFORS AG CH-4103 shaker whilst shaking continuously at 96 rpm,

diffused light and laboratory temperature. A 7-day-old BY-2 cell culture was used for the actual experiments. The experimental set-up (including subcultivation) was carried out in the sterile conditions of a flowbox with standard, single-use serological pipettes. Sterility was maintained by burning the Erlenmeyer flask necks in the flame of a gas burner and immediately sealing them with aluminium foil. The foil was then taped with clingfilm, as shown in Fig. 1. Four experimental variants were chosen to monitor the 2,4-D impact on the BY-2 suspension and the content dynamics of 2,4-D, ACC and ethylene throughout the cultivation in the liquid medium, as shown in Table 1.

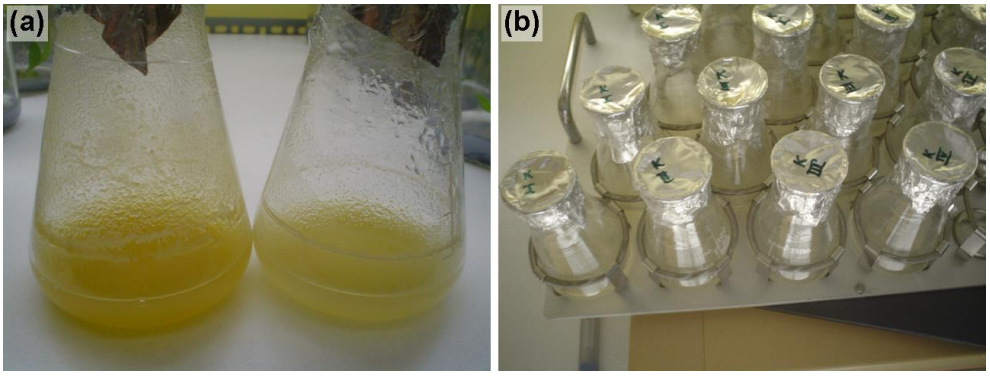

Fig. 1 Erlenmeyer flasks (a) BY-2 tobacco cell suspension, (b) 4 experimental varieties on the shaker.

Table 1. Experimental variants with 2,4-D and different concentrations

Variant	Medium
1.	0.1 μM 2,4-D
2.	1 μM 2,4-D
3.	10 μM 2,4-D
4.	100 μM 2,4-D

2.2. Establishing the growth parameters of the cell suspension

Different growth parameters were evaluated every day for a period of 6 days of culture to assess the impact of different 2,4-D concentrations and their toxicity to BY-2 suspension cells. The parameters were viability, the number of cells and the dry matter weight. The 2,4-D concentration in the cells and the medium, and the 1-Aminocyclopropane-1-carboxylic acid (ACC) and ethylene concentration in the cells; were established from collected and freeze-dried samples every day of culture. A gentle method of sampling was required for the analytical measurements, as the suspension cells mustn't be stressed and damaged. The surface of the cells mustn't have been contaminated. A vacuum separator served these purposes. This separator removes the medium from the suspension while maintaining the viability of the cells and retaining 2,4-D in the cells along the process.

The BY-2 cell suspension viability was tested with specific fluorescent dyes. There were 37 μl of the BY-2 suspension pipetted into Eppendorf micro test tubes, and 3 μl of fluorescein diacetate (FDA) and 10 μl of propidium iodide (PI) were added to distinguish between living and dead cells. Fluorescein (intense green fluorescence) results from the FDA active cells and the hydrolytic reaction of plant esterase. PI doesn't penetrate the living cells, but it does so in the dead cells (red fluorescence of the nuclei of the dead cells) due tothe corrupted permeability of the cell membranes. Triphenyl tetrazolium chloride (TTC) dying was used for a quick check of the suspension's viability throughout the cultivation process. Oxidoreductase

reduces the substrate and the living cells gain a different intensity of the colour pink with the reduced formazan. The representation of the living and dead cells was evaluated by photo documentation using the OLYMPUS AX 70 fluorescent microscope video camera and OLYMPUS SP-350 camera. The viability of the cells was defined in percentages as a ratio of the number of living cells to the total number of cells, multiplied by 100. The results were recorded in a graph.

The density (the amount) of the cells was set to 1 ml of the suspension. The Fuchs-Rosenthalcounting chamber and the OLYMPUS CX31 optical microscope were used to evaluate the suspension growth and to determine the number of cells in the liquid medium. The determined values were inserted in the formula (1):

$$P = p \cdot \frac{1}{o} \cdot z, \tag{1}$$

where P is the number of cells in 1 ml, p is the average number of cells in one square, o represents the volume of one square (Fuchs-Rosenthal chamber=1/80 mm^3), and z is the dilution, that is the sum of the parts.

The suspension samples were collected regularly fromculture day 1 to 6, weighed (fresh weight), freeze-dried, and subsequentlyweighed again (dry matter) in order to determine the weight of the dry matter of the cell mass.

The BY-2 cells were observed with OLYMPUS PROVIS optical microscope. The morphological and structural changes of the cell variants were evaluated every day of culture and photo-documented with the OLYMPUS SP-350 camera.

2.3. Establishing the growth regulators (2,4-D and ACC to ethylene conversion)

The following methods were used for all experimental variants to establish the growth regulators content: extraction, homogenisation, and purification of the cell suspension samples, and 2,4-D quantification in the cells and the medium with the Enzyme-Linked Immuno Sorbent Assay (ELISA) test. Gas chromatography (GC) was used to assess the ACC to ethylene conversion. The separation of the cell suspension from the growth medium was carried out using negative pressure. The medium samples were analysed with ELISA after dilution of the initial sample.

The sample of each variant (1 ml suspension) was taken under vacuum – 25 kPa with the Dorcus vacuum separator (Tessek, ltd, Czech Republic) that facilitates the separation of the medium and the cell mass. The Luer lock syringes were inserted in the adaptors on the lid of the machine with two Whatman filter paper discs. 1 ml of the cell suspension was pipetted into the syringes. The adaptors were set in a position to allow the medium to be taken into the test tube before the water pump was turned on. The obtained filtrate was pushed out of the syringe with a metal stick and the filter paper was removed. The cell mass was then weighed, frozen in liquid nitrogen and freeze-dried. The prepared samples were homogenised in 8 ml of 80 % methanol, and butylated hydroxytoluene (BHT; 100 mg/l) was added as an antioxidant. BHT was not used for ACC determination. The homogenised extracts were stored overnight at the temperature of -20 °C. The next day, the extracts were shaken for 1 hour in the fridge at the temperature of 4 °C and centrifuged for 5 minutes at 5000 g. The supernatants were evaporated to dryness on a vacuum rotary evaporator. The evaporated samples were diluted in

1 ml of distilled water and were sonicated in an ultrasonic bath in an evaporating flask. The purified samples were transferred into micro test tubes and frozen at the temperature of -20 °C.

The principle of the ELISA test (immunoprecipitation reaction) lies in the competitive reaction of the growth regulator (2,4-D) from the plant extract and of the ligand (2,4-D enzyme conjugate) bound to the molecule of a specific antibody. The evaluation was carried out quantitatively by spectrophotometric measurement of the absorbance of the product of the enzymatic reaction after the delivery of the substrate to the antibody-ligand complexes. The microplate was covered in an E2/G2 monoclonal antibody (100 ml per socket) a day before the evaluation and was incubated overnight in a 50 mM concentration carbonate coating buffer (dilution 1:1000) at laboratory temperature. The microplates were emptied the day after and washed with the phosphate-buffered saline with Tween detergent (PBST) at a pH of 7.2. The 2,4-D standards (100 ml, calibration range: 0,1; 0,2; 0,5; 1; 2; 5; 10; 20 ng/ml) were pipetted into the dried microplates. Diluted samples were pipetted as well (100 ml; water dilution 1:100) into 6 wells of 100 ml of distilled water for maximum binding and into 6 wells of 150 ml of PBST for the blank. 50 ml of the 2,4-D/peroxidase conjugate (dilution 1:6000) was then pipetted into all wells, apart from the ones for the blank. The microplates were washed three times with the PBST buffer for 1 hour. Subsequently, 100 ml of the tetramethylbenzidine substrate was pipetted into the wells. The enzymatic colour reaction was stopped after 15 minutes with the application of 100 ml of 1 M H_2SO_4. The colour change is directly proportional to the concentration of the researched substance (2,4-D) in the sample. The optical density of the colour product was read on a SUNRISE colourimeter at a wavelength of 450 nm. This method was performed according to the study by Franek [26].

ACC and ethylene were assessed by gas chromatography with a flame ionization detector (FID) following the publication by Fiserova [27]. The method was carried out in test tubes that were sealed with a rubber stopper to extract the gases with a syringe.

3. Results and discussion

3.1. Evaluation of the morphology of the BY-2 cells suspension in the liquid medium

The morphology of the BY-2 cell suspension cultivated at different 2,4-D concentrations was evaluated with microscopic techniques. The pictures are recorded in Fig. 2. After 24 hours, the cells of all the varieties were capable of mitosis. After 48 hours of cell cultivation, the control variant showed long, one-row multicellular chains, typical for this suspension culture. In this phase, the other variants formed chains as well and didn't differ in morphology and size (small volume). The cell nuclei were distinct in all variants. On day 3 of culture (the exponential phase), the morphological changes and differences between the variants started to manifest. The control cells of 1 μM and 10 μM retained a small volume, kept forming chains, and had distinct nuclei. The lowest concentration showed spherical to oval-shaped cells. At this stage, the cell expansion and separation processes had already started taking place. The size of the cells varied, some cells were already significantly enlarged, and there was a great variety of shapes in the elongated cells, as shown in Fig. 2 (b). The activation of the enzymes, which facilitate these two processes, clearly begins to take place at this stage. The same conclusion was drawn in the study of the *Daucus carota* suspension. The cells continued elongating till day 12 ofculture without the 2,4-D present, while the elongated morphology was not observed in the cells with 2,4-D present even on day 12, as stated in the study by Masuda [28]. The highest activity of β-galactosidase and β-xylosidase enzymes was recorded between days 3 and 4 in the elongating cell of the carrot suspension in the study by Masuda

[29]. At the end of day 6 of culture (Fig. 2 (c)), the control cell variants were greatly enlarged and elongated, and the nuclei of some of the cells were indistinct. The general phenomenon was thus confirmed that as the number of cells increases at the beginning of cultivation, their volume decreases, only to increase later. McCann [30] observed that the carrot suspension cells enlarged up to 20x when cultivated without 2,4-D during the first three days. In the study by Miyazawa [31], the length of the measured cells with 1 μM of 2,4-D reached 43.3 – 45.4 μM between days 2 and 4, while the 2,4-D-free cells were already extremely elongated, reaching 135 – 154.7 μM. This distinct elongation phenomenon was only confirmed for low 2,4-D concentrations. Toyooka [32] also observed the morphological changes in the BY-2 transformed cells both in the exponential and stationary phases. In the exponential phase (days 3 and 4 of culture), the cells had several media to large-sized vacuoles, usually separated, some of which fused. Organelles such as peroxisomes, mitochondria, and plastids were distributed throughout the cytoplasm. Golgi stacks, multivesicular bodies, trans-Golgi network, as well as secretory vesicle clusters were widely spread throughout the cytosol. The cell length ranged around 50 μM in the exponential phase and approximately 60-100 μM in the stationary phase (days 6 to 7). In the stationary phase, the elongated cells contained large vacuoles of 50-100 μM, and the aforementioned compartments lay in a thin layer between the vacuoles and the cell wall.

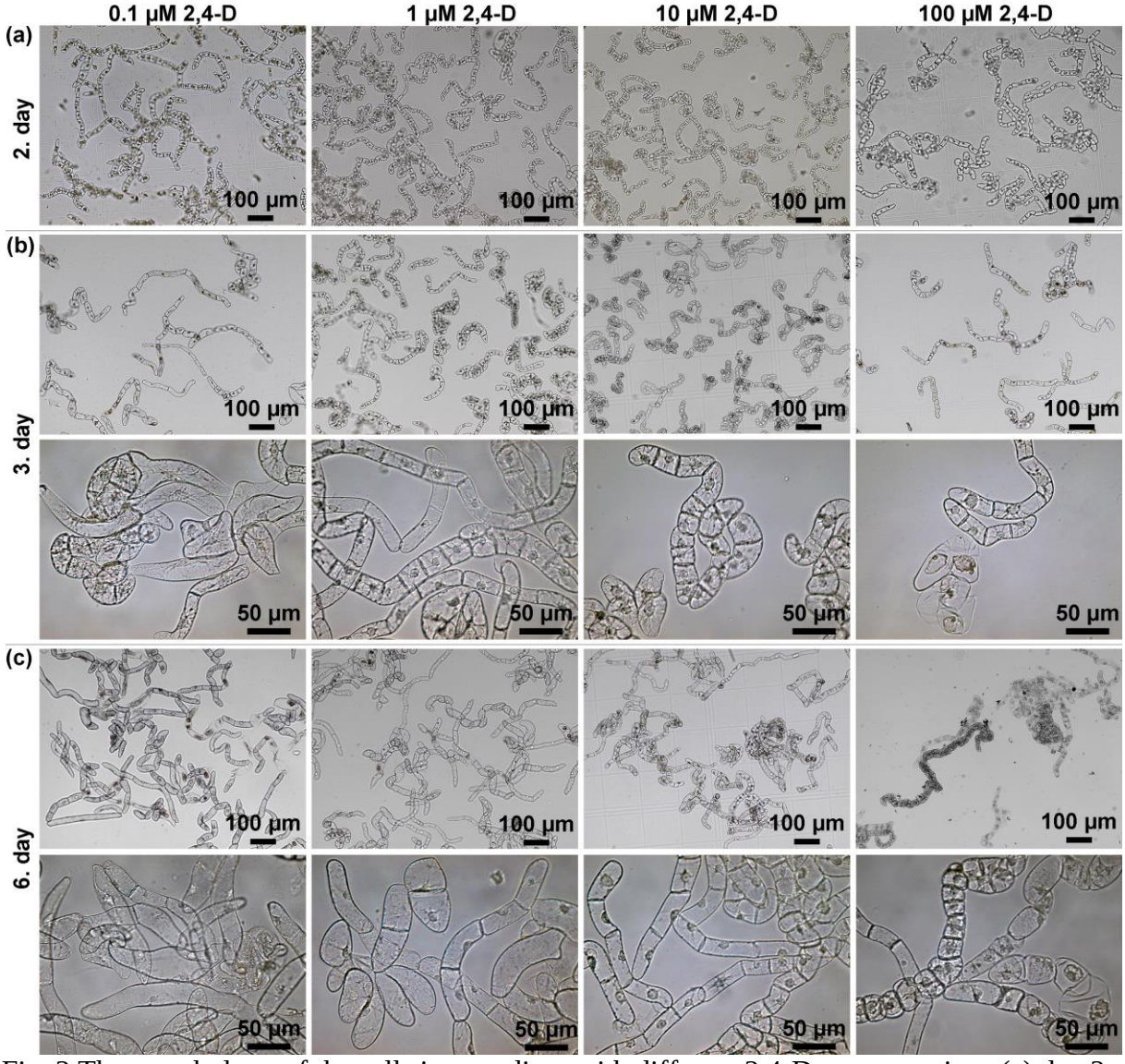

Fig. 2 The morphology of the cells in a medium with different 2,4-D concentrations (a) day 2 of culture, (b) day 3 of culture, (c) day 6 of culture.

In the stationary phase, the cells of 10 µM of the 2,4-D variant remained organised in chains, retained a small volume, and had distinct, round nuclei. The cell size didn't change significantly since the beginning of thecultivation. At the lowest concentration, the cells were already substantially enlarged and elongated, and their nuclei were indistinct due to the lack of 2,4-D. The cell wall was often very thin in comparison with the other variants. After exposure to the highest 2,4-D concentration of 100 µM, the cells were often dead. The surviving cells showed heavily thickened cell walls and compartments; but remained organised in chains or formed multicellular clusters. The observed cytoplasm was condensed and brown. Mitosis occurred in a non-standard way, and the cells formed double-row chains (Fig. 3 (a)). The long-acting toxic 2,4-D concentration caused the change in the cell compartment orientation, which could be related to the polarity reversal. The same double-row chains were observed in BY-2 cells transformed with the *Spcdc*25 yeast gene for the Cdc25 phosphatase. The Cdc25 phosphatase is a crucial enzyme for the dephosphorylation process and mitosis regulation. Unlike the control variant, which only formed single-row chains, the largest fraction of doublets (up to 34 %) was detected on day 4 of culture. The study by Orchard [33] proved that the *Spcdc*25 gene induces cell division either transversely,

or longitudinally. In the same cells that were transformed by the *Spcdc*25 gene, the presence of 1 mg/l^{-1} of 2,4-D and 1 mg/l^{-1} of NAA with the addition of 1 mg/l^{-1} of 6-Benzylaminopurin (BAP) cytokinin in the medium stopped the typical chain formation in the cell suspension from the derived tobacco plant (*Nicotiana tabacum* L., cv. Samsun). The cells clumped together, or formed doublets, as found in the study by Suchomelová-Mašková [34]. This means that the *Spcdc*25 alien yeast gene has a similar effect to the one of a strong toxic concentration of 2,4-D 100 µM. Throughout the cultivation, we noticed a massive impregnation and accumulation of amorphous material in the cell walls and their thickening depending on the 2,4-D concentration. An evaluation of this parameter, however, requires differentiation between the living and the dead cell in the suspension. Atthe end of the cultivation, we observed that the BY-2 cells decrease their volume depending on the concentration, as visible in Fig. 3 (a). Photographs of morphological changes in different stages of apoptotic-like programmed cell death (AL-PCD) were taken throughout this study and are depicted in Fig. 3 (b). Features typical for AL-PCD in most cells were recorded on day 9 of culture of the control variant of BY-2 suspension. Both the early stage of separation of the cytoplasmic membrane from the cell wall and the fully condensed cytoplasm were captured. The nuclei of the living cells were located at the edge of the cell walls, which maintained their integrity.

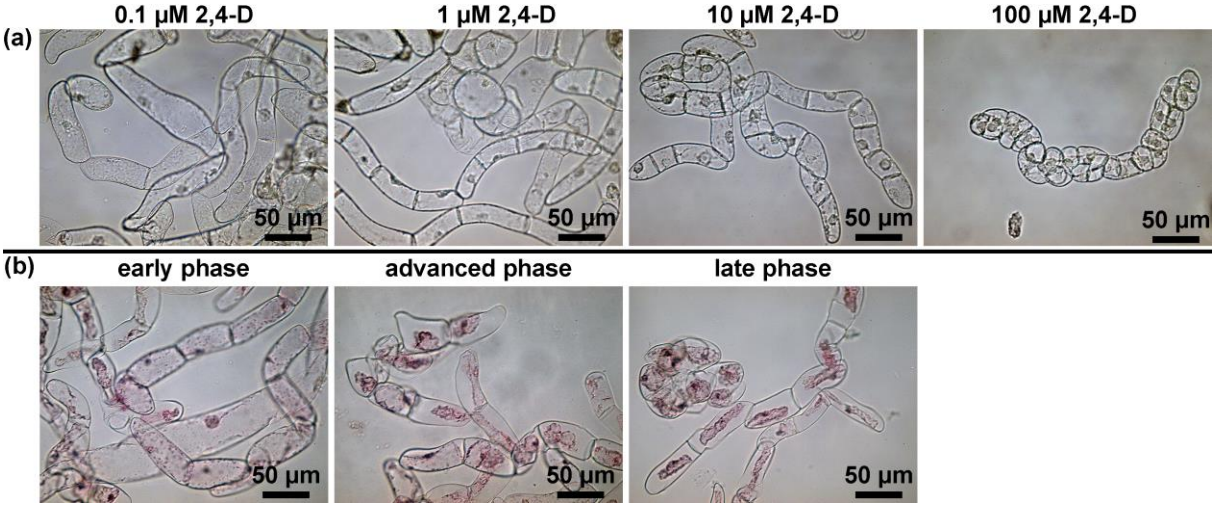

Fig. 3 (a) Morphology of the cells on day 6 of culture in medium with different 2,4-D content, (b)Morphological changes in different AL-PCD phases, maintenance culture 1 µM of 2,4-D, day 9 of culture, dyed with 200 mg of Tetrazolium chloride (TTC) to 25 ml of suspension.

3.2. Determination of the number of BY-2 cells in the suspension and their viability in the liquid medium

The 2,4-D influence on BY-2 cell suspension was established every day of culture with the following parameters: the number of BY-2 cells in the suspension (see Fig. 4 (a)) and their viability in the liquid medium, as shown in Fig. 4 (b).

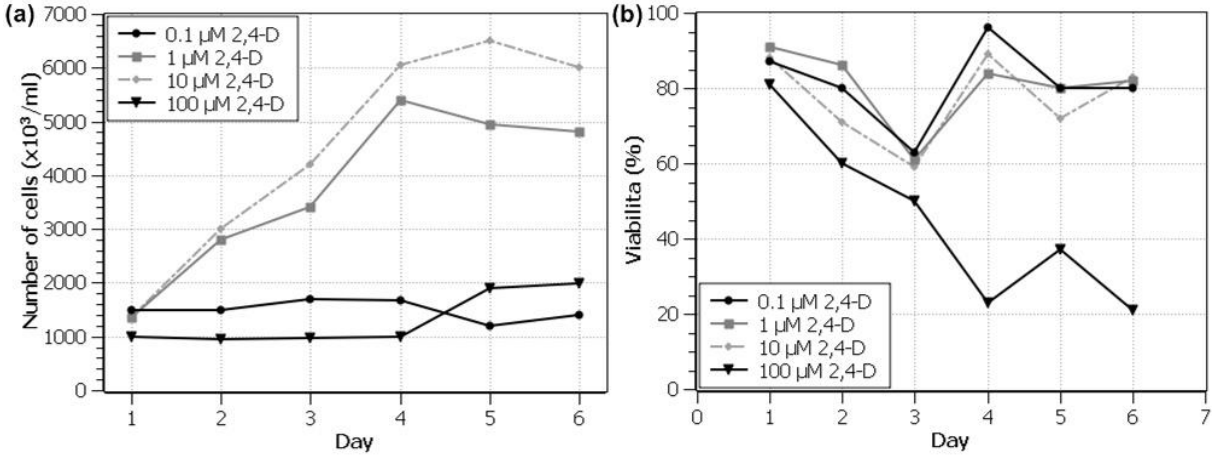

Fig. 4 (a) BY-2 suspension growth in relation to the 2,4-D concentration in the liquid medium and to the exposure period, (b) the viability of BY-2 cells in the liquid medium with different 2,4-D concentrations.

The number of the cells was low – $1500 \cdot 10^{-3}$/ml – at the start and the end of cultivation in the suspension cultivated in a medium with the lowest 2,4-D concentration of 0.1 µM; and didn't change significantly throughout cultivation. The number of cells was also low – $1000 \cdot 10^{-3}$/ml – in the medium with the highest concentration of 100 µM. It began to increase slightly towards the end of days 5 and 6 of culture. The 1 µM of the 2,4-D control variant continued to grow, as was expected. Within four days, the number of cells tripled to about $5{,}500 \cdot 10^{-3}$/ml, followed by a decline in growth on day 5. The number of cells in 10 µM of the 2,4-D variant quadrupled within 5 days up to $6500 \cdot 10^{-3}$/ml, followed by a slight decline on day 6. The experiment didn't validate that the commonly used 1 µM of 2,4-D control variant delivers the highest growth efficacy. The same was corroborated in the study by Campanoni [35], which determined the most effective concentration to be 10 µM for the cell division growth of the tobacco suspension VBI-0. More so, it confirmed that this concentration inhibits the cells' elongation. The measured values of our experiment also correspond with the evaluated cell morphology. The highest 10 µM variant showed well-observable, intact, round nuclei on day 6, and it kept the cells in long chains even at the end of culture. The differences in cell numbers between the variants were significant, as evident from the graph in Fig. 4 (a). For the purposes of this experiment, the 0.1 a 100 µM of 2,4-D concentrations can be described as non-growth, where 0.1 is viable and 100 µM is lethal. The control variant of 1 µM and 10 µM of 2,4-D concentrations can be described as growth concentrations (growth active). The ratio of living and dead cells was evaluated along with the number of cells for every day of culture, expressed as a viability percentage (see Fig. 3 (b)). The viability of the cells ranged between 80 to 90 % after several hours of subcultivation in liquid media with four experimental 2,4-D concentrations. An increased number – about 40 % – of dead cells was recorded after only 24 hours of cultivation in the 100 µM variant. The viability of the cells decreased significantly after 48 hours of cultivation in all variants, ranging between 50 and 64 %. In the following cultivation days, the viability of the culture in the highest 2,4-D concentration medium kept decreasing significantly, dropping to 22 % at the end of cultivation. The number of living cells over dead cells predominated in the other variants, reaching about 80 % even in the stationary phase. The culture with a higher concentration of 10 µM of 2,4-D maintained relatively high viability. The changes in viability were recorded in the following microphotographs in Fig. 5. The plasmatic membranes of BY-2 cells exposed to 50 µM and 100 µM of aluminium lost their integrity 18 hours into its application in the medium. At the same time, there was a significant increase in the vacuolar processing enzyme (VPE) and the vacuolar cell collapse, as presented in the study by Karyia[36].

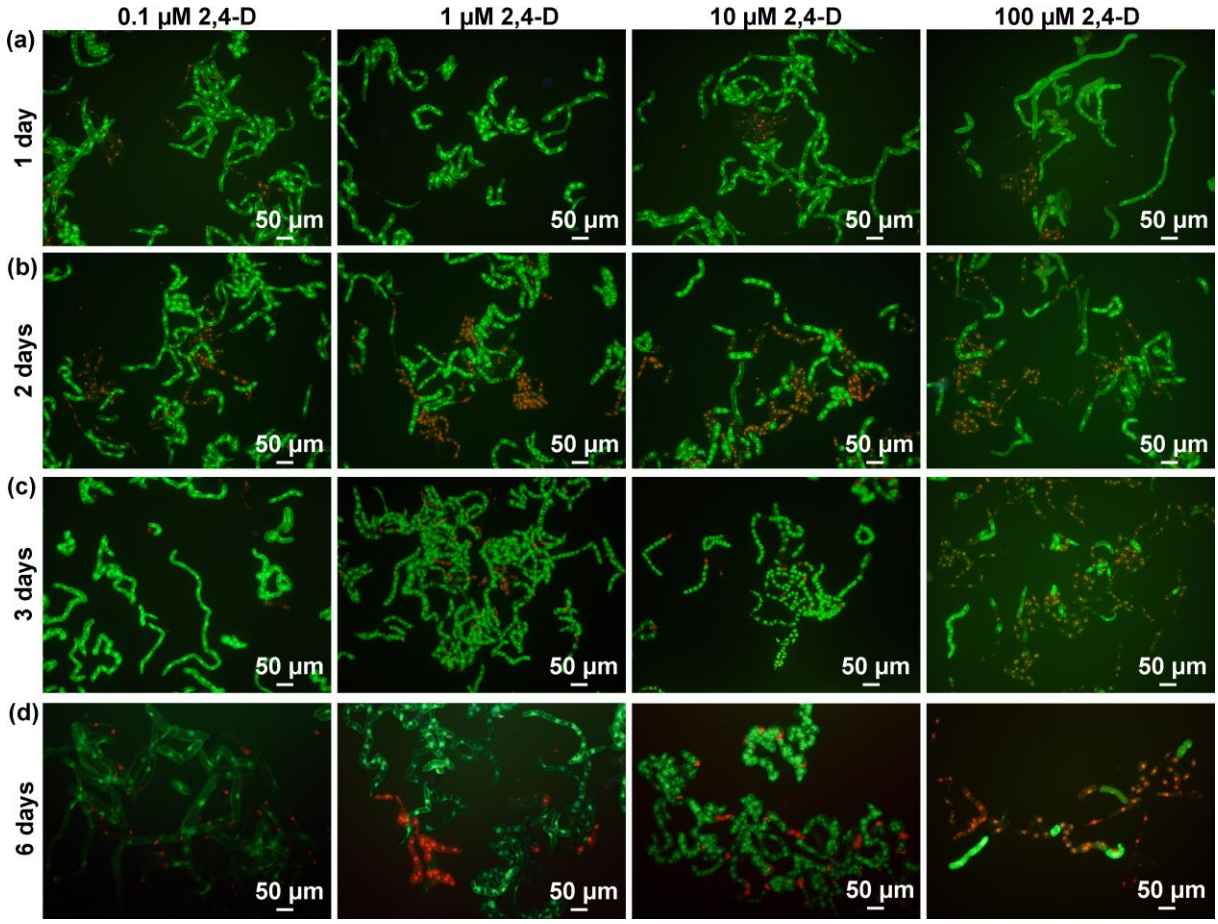

Fig. 5 Microphotography of BY-2 cells in liquid medium for viability determination (a) 1 day, (b) 2 days, (c) 3 days, and (d) 6 days.

The tobacco cells that were exposed to chemical stress released volatile organic compounds (VOCs) with an antioxidant effect (methanethiol, dimethyl sulfide and dimethyl disulfide). The inducers of the cell death were 5 µM biotic nature Mastoparan (MP, wasp venom), 5 µM Camptothecin (CPT, alkaloid), and the abiotic stress agent 100 µM CdSO4. These three chemicals induced an apoptotic-like phenotype PCD that graduated up to 100 % dead cells within 56 hours. The cell death ranged between 15–35 % after 14 hours and 30–55% after 28 hours. The cytoplasm was shrunk and the nucleus condensed in the dead cells. These PCD characteristics were observed for all stress chemicals mentioned in the study by Iakimova [37]. In the study by Kobylińska [38], the BY-2 cells were cultivated over a period of 8 days and exposed to the flavonoid quercetin in different concentrations (0.001, 0.01, 0.1 and 1 mM). The results showed that a dose of 0.1 mM of quercetin stimulated cell proliferation and maintained cell viability, while the highest concentration of 1 mM stopped the cell cycle in the G2 phase and led to cell death. The application of 1 mM during the lag phase dramatically increased cell mortality and, by the end of the experiment, all the BY-2 cells were dead. When applied during the exponential phase (day 4), the effect was a lot milder; only 34 % of the cells were dead by the end of the stationary growth phase with an equal concentration. The results indicate that quercetin was less toxic when added in the exponential growth phase even in high concentrations.

3.3. Determination of other parameters of the studied material

The percentage evaluation of the dry matter characterises the changes in water content in the cell mass over the cultivation period. Over the course of the cultivation, the dry matter weight decreased with the low concentrations, stagnated at 10 μM of 2,4-D, and increased with the highest concentration of 100 μM of 2,4-D. There was a slight decrease on day 6 of culture, as shown in Fig. 6. The change in the cell volume, and hence also the decrease and increase of the percentage of the dry matter content in the cell suspension at different 2,4-D concentrations can be explained by the fact that 2,4-D seriously pectinases the cell walls. This becomes clear from the cell morphology photo documentation. If pectin starts to accumulate too early, it mechanically prevents the cells from reaching their normal size. At high concentrations, the cells remain small, and the water content decreases.

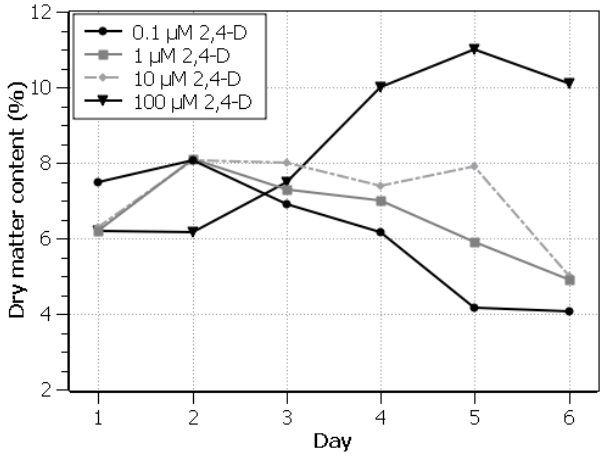

Fig. 6 Determination of the percentage of the dry matter content of BY-2 suspension.

Other observed parameters of the cell medium during generation time were ethylene and ACC (1-Aminocyclopropane-1-carboxylic acid) concentrations. Fig. 7 (a) depicts the ACC concentration changes during generation time. The value of ACC at 'Day 0' represents the ACC concentration at the point of cultivation establishment. It is identical for all 4 monitored variants. This value decreased for 0.1, 1, and 10 μM concentrations because it was spent on ethylene synthesis. From 'Day 2' on, the concentration stayed at its minimum, meaning that all forming ACC was used to synthesise ethylene. The high concentration of 100 μM of 2,4-D in the medium cumulated ACC in the cells till the end of the culture. The steep increase during the first day indicates that the cells produced more ACC than they could metabolise. Fig. 7 (b)representschangesinthe ethylene concentration in the medium. This means that 2,4-D affected the ethylene metabolic pathway. All the viable 2,4-D concentrations stimulated ethylene synthesis. The highest increase was seen in the viable, but non-growth lowest concentration of 0.1 μM, as shown by the line of the graph. The measured values show a steep increase in ethylene from cultivation day 3 in 0.1, 1 and 10 μM of 2,4-D concentrations. The 100 μMconcentration produced the ACC ethylene precursor, which accumulated in the cells. However, a lot less was converted into ethylene. The ethylene level remained low and constant throughout the generation time. The study by Liang [39] proved that silicon (2 mM K_2SiO_3) distinctly increased ethylene production; and induced BY-2 cell death when the aminooxy acetic acid (AOA) inhibited the ethylene synthesis. Silver nitrate (20 μM $AgNO_3$) – another ethylene inhibitor – generated H_2O_2 and nitric oxide (NO) production, and, in the end, led to cell death. The cell fresh weight and viability plummeted after NaCL + Si + Ag treatment, which means that ethylene production is vital to the tolerance to salt stress.

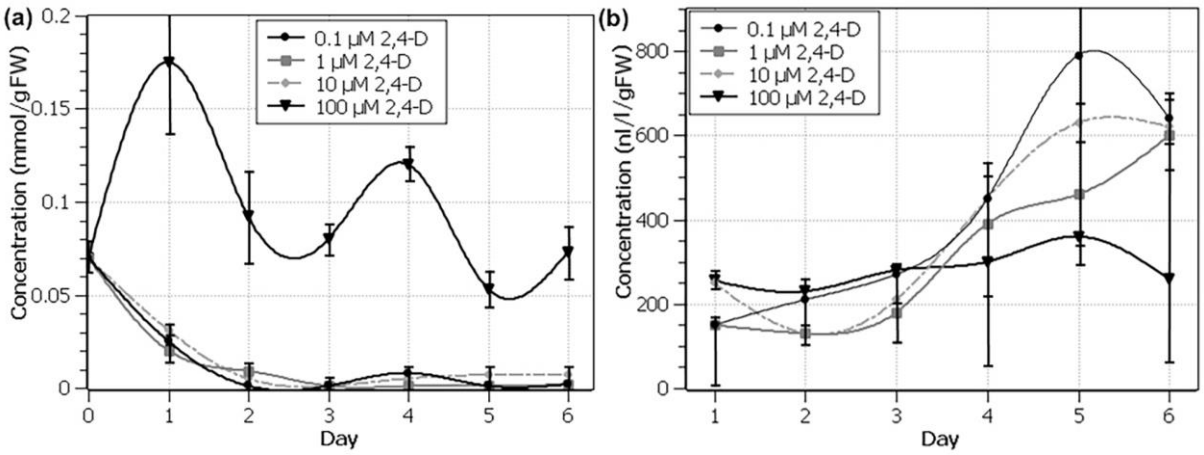

Fig. 7 Determination of (a) ACC and (b) ethylene concentration in the samples throughout the experiment.

The 2,4-D concentration was monitored both in the cells and in the cultivation medium suspension throughout the generation time. The compiled results are shown in the graphs in Fig. 8. The depicted graphs show that, at the lowest concentration of 0.1 µM, the 2,4-D content in the cells stayed at minimum levels throughout the cultivation. The 2,4-D content was increasing till day 3 of culture for the other concentrations, and roughly from day 3, its concentration in the cells started decreasing, as evident from the graph in Fig. 8 (a). At high concentrations, a significant amount of 2,4-D remained in the medium (Fig. 8 (b)). The effect of silver ions on the plasma membrane of the plants is largely connected to the ethylene signalisation inhibition because of the silver ions' ability to replace copper cofactor in the ethylene receptor. The 10 µM $AgNO_3$ exposed BY-2 cells began losing water 10 minutes into the experiments and plasmolysis was taking place. Uptake and accumulation of [^{3}H] 2,4-D or 1-naphthalene acetic acid [^{3}H]NAA in cells was inhibited in the presence of $AgNO_3$ in the medium, compared to the control variant, as cited in the study by Klíma [4].

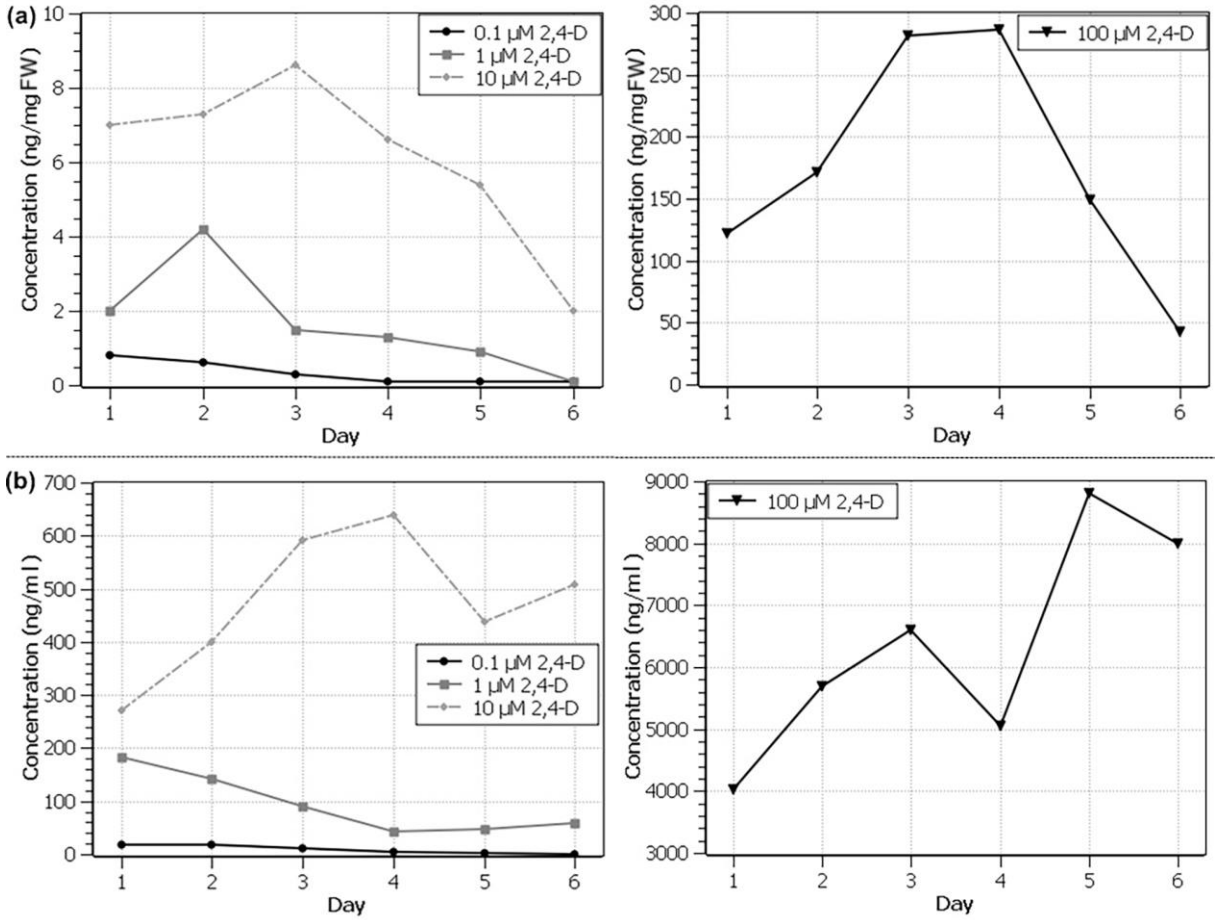

Fig. 8 Determination of 2,4-D concentration (a) in the cells in the experiment, (b) in the culture medium solution.

3.4. Pectin shield hypothesis

Throughout the cultivation, we noted that the accumulation of the amorphous material in the cell walls and their thickening occurred depending on the 2,4-D concentration in the liquid medium (see Fig. 9). Fig. 9 (a) shows the original BY-2 (‘Bright Yellow’) mother culture of the tobacco suspension that was used for the experiment. The documentation took place in the stationary phase on day 6 of culture. It shows elongated cells, but not significantly thickened cell walls. Fig. 9 (b) depicts the cell morphology on day 2 of culture in the established experiment with different 2,4-D concentrations in the live medium. All concentrations at the end of the lag phase show significantly thickened cell walls in all cells. Fig. 9 (c) depicts the cell morphology at the end of the cultivation in the stationary phase. The morphological change in the cell walls’ thickness and compartment areas is visible. The accumulation of the amorphous material in the cell walls and their thickening correspond with the 2,4-D concentration during cultivation. At the same time, we can easily observe that the cells decrease in volume as a function of the 2,4-D concentration in the medium.

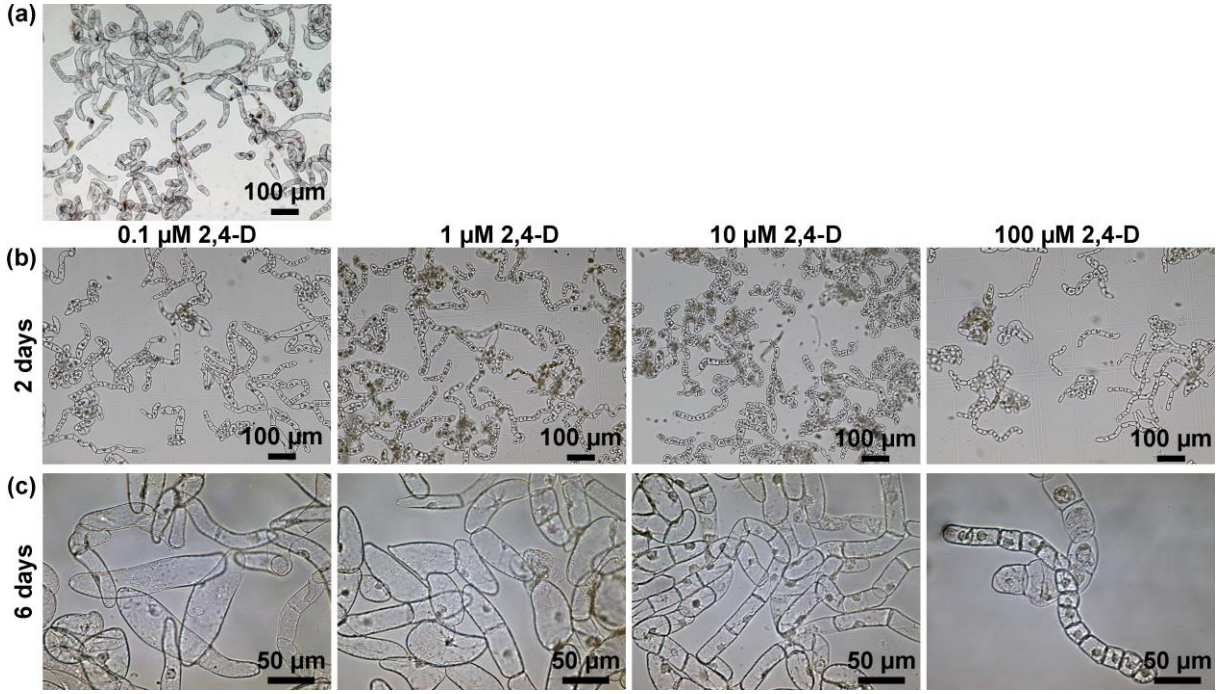

Fig. 9 Cells' morphology in the medium with different 2,4-D concentrations (a) regularly passaged mother culture – control variant (1 µM) on culture day 6, (b) 2 days, (c) 6 days – detail.

Thus, we theoretically assume that 2,4-D influences the massive synthesis and the accumulation of protective pectin substances (hemicelluloses) in the cell walls. The intensive shaking of the suspension, the presence of sucrose and calcium ions in the medium, the acidic pH of the medium, and the degree of esterification of the main pectin component – galacturonic acid, even water itself can be the main factors which encourage the pectin's natural gelling properties. Cell wall remodelling is also mentioned in the studies by McCann [41] and Kieffer [42]. A significant decrease of two degrees in the pH on day 2 of culture and a distinctly elevated level of uronic acid were also confirmed in the study by Issawi [43]. In his study, Hensel [44] claims that for the tobacco cells of the S-2 line, the content of the main components of galacturonic acid pectins reached its maximum on day 2 of culture with 0.22 $mg \cdot l^{-1}$ of 2,4-D and 0.19 $mg \cdot l^{-1}$ of NAA. It was 50 % higher than in the second variant, where the synthetic auxin was missing in the medium, and the decrease of the pectin levels continued till the end of the culture (day 10). The cellulose content was 50 % higher with 2,4-D present at the beginning of the cultivation (day 0), comparable on days 1 and 2 for both variants and plummeted on day 4. Day 2 of culture was also followed by a significant drop in pH for both variants from 5.3 to 4.7. Furthermore, pH increased rapidly in the absence of 2,4-D and increased more slowly in its presence. The chaining process mechanism (formation of characteristic multicellular chains) can be explained by the fixation of hardened pectins in the hemicellulose network of the cellulose skeleton of the cell walls and by the drastic pectination of cell compartments. The formed polysaccharide pectin (or hemicellulose), whose function is to strengthen the cell wall, inhibits intussusception (cell growthby elongation). Therefore, if the effect of pectin prevails over auxin, intussusception does not take place, but if the effect of auxin prevails over pectin, then intussusception does take place. The synthesis of pectin in plants is known to be triggered by stress conditions, as presented in the study by Colzi [20] or Lherminier [45]. If the pectin shield hypothesis is proven in artificial conditions, we can consider the foreign, synthetic 2,4-D substance to be a stress factor – stressor. The BY-2 suspension is one of the few cultures to have an excellent defence system thanks to its ability

to form protective substances. Hybrid mint (*Mentha arvensis cv.sanbi x M.spicata car. Crispa*) also exhibits these morphological properties (the formation of multicellular chains), as presented in the studies by Krzyzanowska [46] and Yang [47,48]. 2,4-D promotes a heavy production of ethylene, which is a stress hormone in plants. If further experiments confirmed the hypothesis of synthesis and accumulation of pectins, the theory could be further developed. The ethylene produced during cultivation can activate pectinase degradation enzymes, which further cleave and break down solidified pectins. The experiments confirmed that the multicellular chains break-separate into individual cells, especially at lower concentrations of 2,4-D in the liquid medium, which accumulated a larger amount of ethylene. If the pectin hypothesis proved true, then the morphology of the experiment allows the 1 μM of 2,4-D concentration to be designated as high and stressful. The theory of uncontrollable BY-2 growth resembling tumour cells in animals would be completely legitimate. Stress conditions in plants promote the synthesis of pectin substances. As presented in the study byLherminier [45], pectins are not only a defence mechanism for plants: in humans, pectins have a beneficial effect on cholesterol levels and counteract poisoning by toxic metal cations, which they bind to themselves, as presented in the studies by Gómez [49] or Zhexenbay [50]. They are also widely used for diarrheal diseases treatment. It is possible that, just like heavy metals, they can bind 2,4-D in a human organism.

The ability to bind divalent and trivalent heavy metals is a key quality for pectin substances, as presented in the study by Mehes-Smith [22]. This is made possible by the carboxyl group that pectins carry. Pectins compensate for the heavy metals in the cell wall, prevent them from penetrating cytoplasm, and hence increase the plant's resistance to heavy metals in the environment. If further experiments confirmed the pectin hypothesis, the defence mechanisms as well as the formation of defence substances in monocotyledons (which are usually not affected by 2,4-D) should be investigated. The pectin shield should not allow 2,4-D to penetrate the cells of these plants, even though its gelling properties are not supported in nature. In natural conditions, auxin supports the expandability of the cell wall when plastic elongation occurs. This change is followed by the synthesis of polysaccharides in the cell wall. However, the order of these events depends on the amount of auxin acting on the cell. Apart from the stress reaction to auxin concentration, pectins form naturally in the cells. This formation happens mainly in the earliest development stages, and pectins are a natural part of the cell wall structure (they constitute about 30 % of the dry matter). In natural conditions, the cells cannot elongate without pectins. Pectins allow the cell to expand plastically in the presence of auxins. If cells' intussusception was prevented in artificial conditions, it would confirm the theory that, at high 2,4-D concentrations in the medium in particular, the synthesis and the polysaccharides' consistency change take place first, possibly with a thickening of the hemicellulose skeleton, which in turn prevents the elongation growth of the cell wall. Thus, higher auxin concentrations have the opposite effect on the cell wall than low concentrations. We can therefore assume that the formation of pectins in artificially created conditions will be enormous. The reduced cell volume also means that there will be a smaller absorption area for toxic substances. We can also assume that pectins can bind not only the aforementioned heavy metal ions in the cell wall; but also a range of other harmful substances, such as dangerous active ingredients of pesticides and other xenobiotics.

A cell in a toxic environment either undergoes apoptosis or converts into a supercell – an "immortal cell". The formation of protective substances at all monitored concentrations of 2,4-D reveals that the substance itself creates stress conditions, that is, a toxic environment for the plant cells. There is, therefore, no minimum amount of 2,4-D that would not be toxic for living organisms. At this point, we can seriously consider the anomalous uncontrolled cell division, plant mutagenicity and supercell production we expected. It is important to emphasise that the BY-2 cells are modified twice with 2,4-D: in callus formation for the first

time and in the liquid medium for the second time. Unlike the organised, intact tissues of intact plants, callus is known to be genetically variable and unstable. The genetic variability/instability of callus cells are characterised by a change in the number of chromosomes. Both the callus cells and the regenerated plants derived from the callus showed variations in the number of chromosomes, as presented in the study by Ogura [51]. Doležel [52] states that there were highly caryologically unstable callus and suspension cultures observed for different species in the medium with 2,4-D. Another study by Doležel [53] proved that the tested growth medium didn't have any effect on the mutation induction in the *in vitro* cultivated cells of *Tradescantia.* However, there was IAA natural auxin (indole-3-acetic acid) used in this medium. Abnormalities in somatic embryos caused by 2,4-D-induced genetic changes in DNA are also described in the study by Garcia [54].
The results led to the conclusion that the 2,4-D content in the cells grew up until days 2 to 3 of culture (see Fig. 8 (a)) and there was a significant substance residue in the medium (see Fig. 8 (b)). In the culture days that followed, it no longer accumulated in the cells. We observed a delayed but similar trend in the graph in Fig. 7 (b). In reaction to the increased 2,4-D amount, the content of ethylene as a stress phytohormone kept increasing up to day 5 of culture in all concentrations apart from the lethal 100 μM. Ethylene stimulates the activity of the enzymes, which hydrolyse polysaccharides, and should hence participate in the cell chains' breakdown (this happens at low 2,4-D concentrations). With the massive accumulation and predominance of the pectin substances, the activated pectinase doesn't seem to be enough to act in the thickened cell compartments and the chains remain. This theory is confirmed in the graphs in Fig. 7 (b). The 1 μM concentration produces more ethylene than the 10 μM 2,4-D concentration. At 10 μM, the living cells' chains don't break at the end of the growth curve, and the cells remain organised in chains. At the high 100 μM of 2,4-D concentration, the surviving cells stay organised in chains at the end of the culture. The ethylene cumulation, however, is much lower than in the other 2,4-D concentrations due to the high cell mortality. It is crucial to visualise and quantitatively determine the polysaccharides of the pectin substances, or hemicellulose, in the BY-2 tobacco cells. That is in the four 2,4-D concentrations, without 2,4-D, as well as in the BY-2 cells callus-derived cells with the natural IAA auxin, to confirm the hypothesis.

4. Conclusions

The presence of the 2,4-D synthetic auxin in the medium is imperative for long-term BY-2 suspension viability. The exact mechanism of the symbiosis of the suspension and the synthetic auxin and the BY-2 cells' organisation in chains remains unclear. This study examined the correlation between 2,4-D and ACC accumulation in the cells, and their ethylene production over the course of 6 days of culture with 0.1, 1, 10 and 100 μM of 2,4-D in the medium. The following conclusions were reached based on the performed analyses:
– 4 studied 2,4-D concentrations had 3 different effects: 1 μM and 10 μM proved active and viable, 0.1 μM non-growth, but viable, and 100 μM non-growth and lethal,
– at the lowest concentration of 0.1 μM, the 2,4-D content in the cells stayed at minimum levels throughout the period of 6 days of culture. In contrast, in other concentrations, the 2,4-D content increasedat the beginning of culture, but decreased from day 3 onward,
– at high concentrations of 10 and 100 μM, a significant amount of 2,4-D remained in the medium,

– the results of this study didn't confirm the general opinion that the 1 μM of 2,4-D control variant produces the most efficient growth. It was the 10 μM of the 2,4-D variant that showed the highest growth in the cell population,
– the phenomenon of the cells decreasing in volume at the beginning of culture and increasing it through an elongating growth of the cell wall later was only confirmed for low concentrations of 0.1 and 1 μM of 2,4-D,
– the changes in the cells' volume were proven by testing the dry matter content percentage, which was decreasing for 0.1 and 1 μM of 2,4-D, constant for 10 μM, and increasing for 100 μM,
– the high concentration of 100 μM showed signs of AL-PCD and produced double-row chains in the stationary phase,
– all the 2,4-D concentrations showed morphological changes in the cell walls and compartments. These were significantly thickened at the beginning of culture in all cells, and all the cells of all variants were organised in filaments. However, on day 6 of culture, only high concentrations of 10 and 100 μM of 2,4-D showed non-elongated cells, with an abnormally thickened cell wall, organised in chains,
– a massive production and accumulation of natural protective substances in the cell walls is expected depending on the 2,4-D concentration in the medium,
– the cell chains can be explained by the cell wall and compartment impregnation through heavy pectin production. Pectin substances change their structure in the artificial conditions of the liquid medium, which encourages their natural gelling properties and the gelation process through its content and its mechanical movement,
– the observed formation of a thick defence substances shield in the cell walls supports the theory of uncontrolled cell division, plant mutagenicity and supercell production,
– an enormous production of the stress phytohormone ethylene was confirmed for the application of the 2,4-D synthetic substance in all the viable variants. All ACC was used for ethylene synthesis. Only the lethal 100 μM concentration accumulated ACC in the cells and produced less ethylene than the other variants.

We can conclude that, based on the discovered facts, this foreign substance creates stress conditions and a toxic environment for all plant cells at all concentrations; and there is no minimum amount of 2,4-D that would not be toxic.

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Conflict of Interest:
Authors K. Muselikova and K. Mouralova declare that they have no conflict of interest.
Author Contributions Statement:

Katerina Muselikova: Conceptualization, Methodology, Data curation. Katerina Mouralova: Writing- Original draft preparation, Writing- Reviewing and Editing, Supervision, Funding acquisition.

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Dear Editor,
I am sending the *Synthetic auxin herbicide 2,4-D and its influence on a model BY-2 suspension* for issue of your journal. Authors of the manuscript are K. Muselikova and K. Mouralova.

The paper is original and data were not published previously.

With regards

Katerina Mouralova

Declaration of interests

x☐The authors declare that they have no known competing financialinterestsor personal relationships that could have appeared to influence the work reported in this paper.

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Fig. 1

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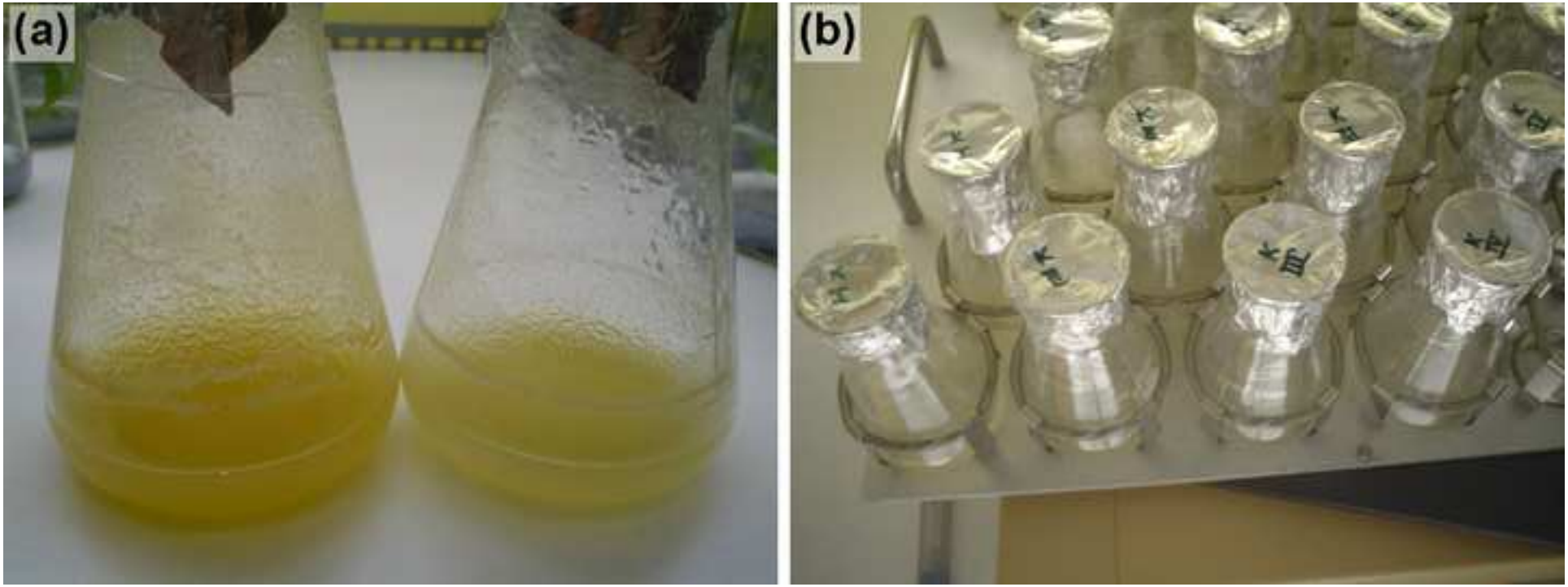

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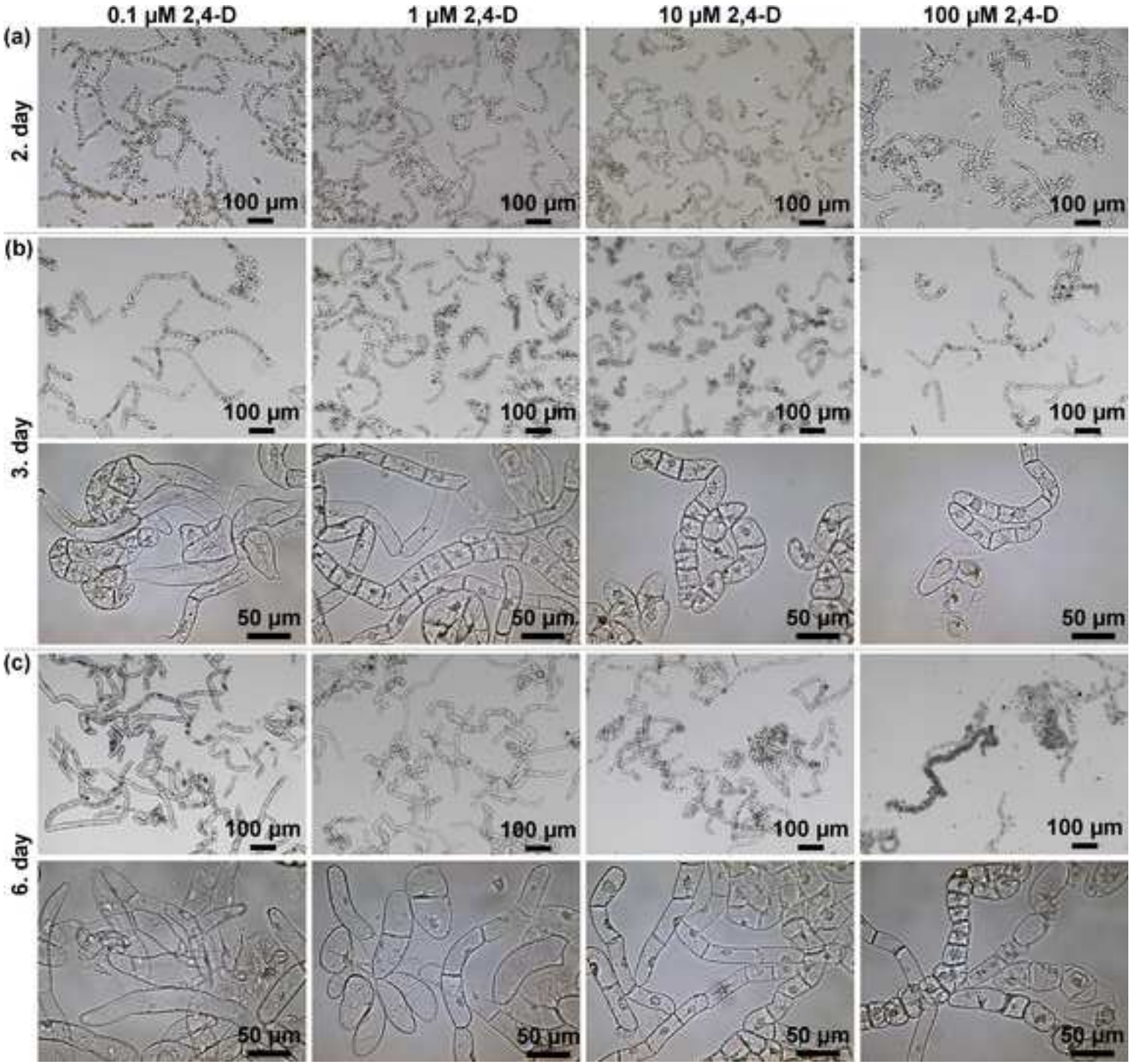

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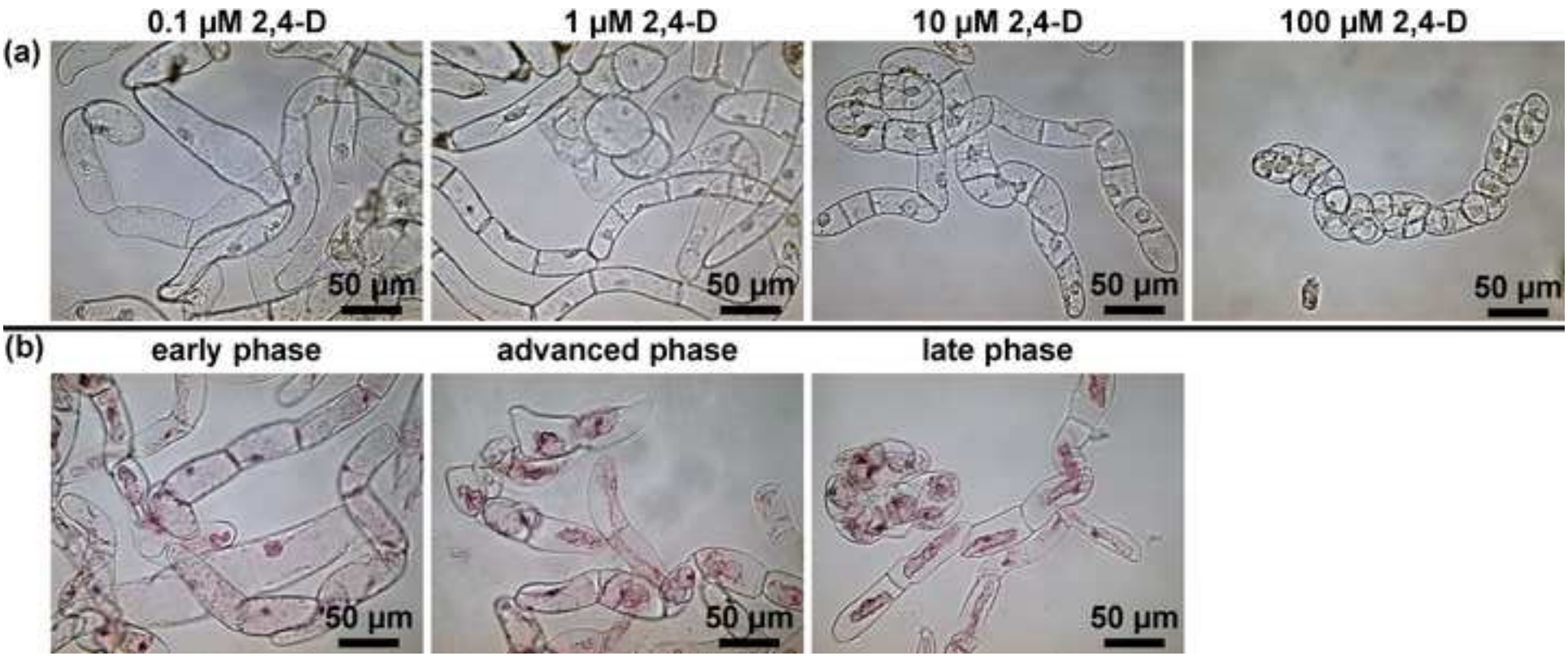

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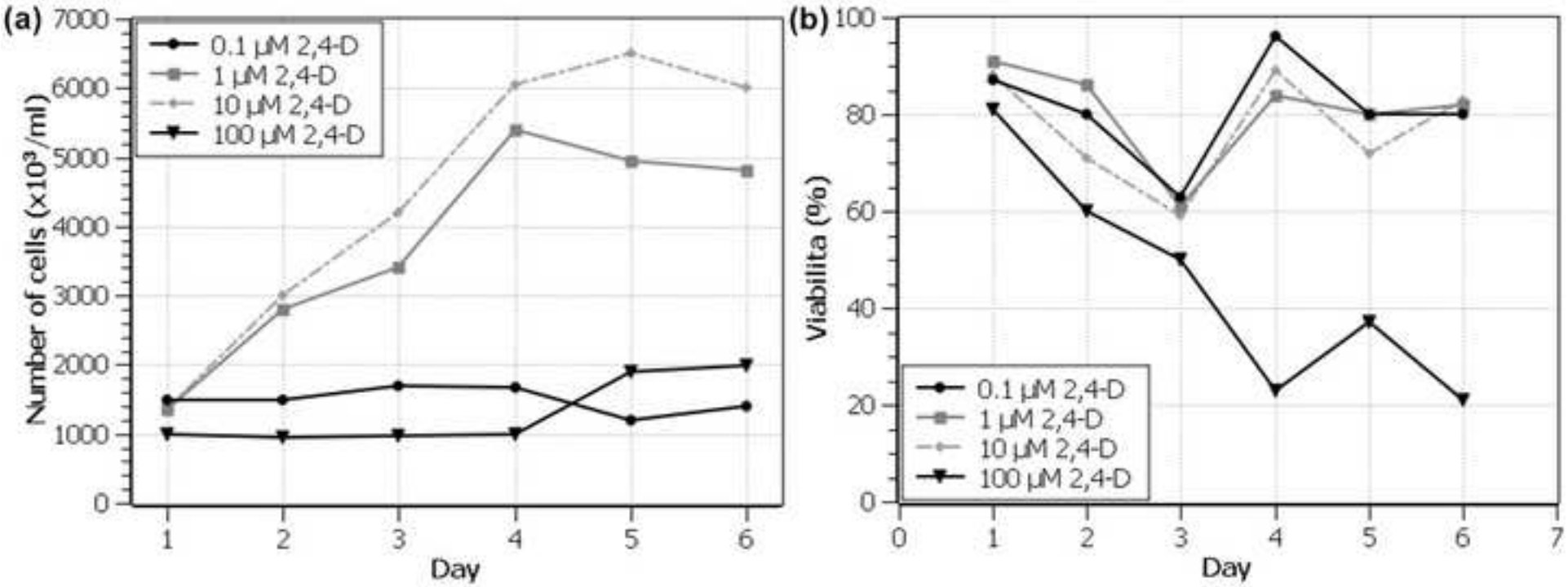

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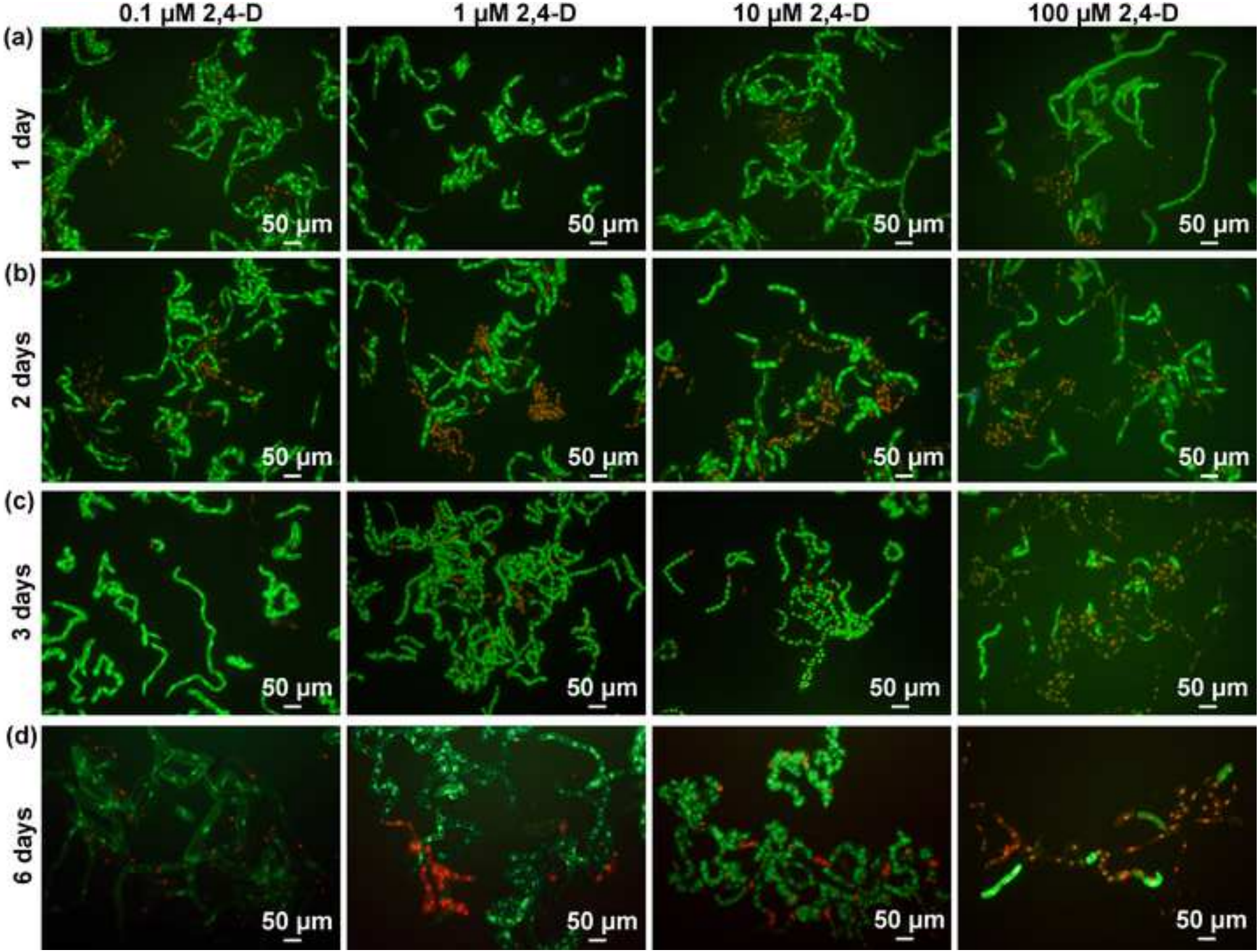

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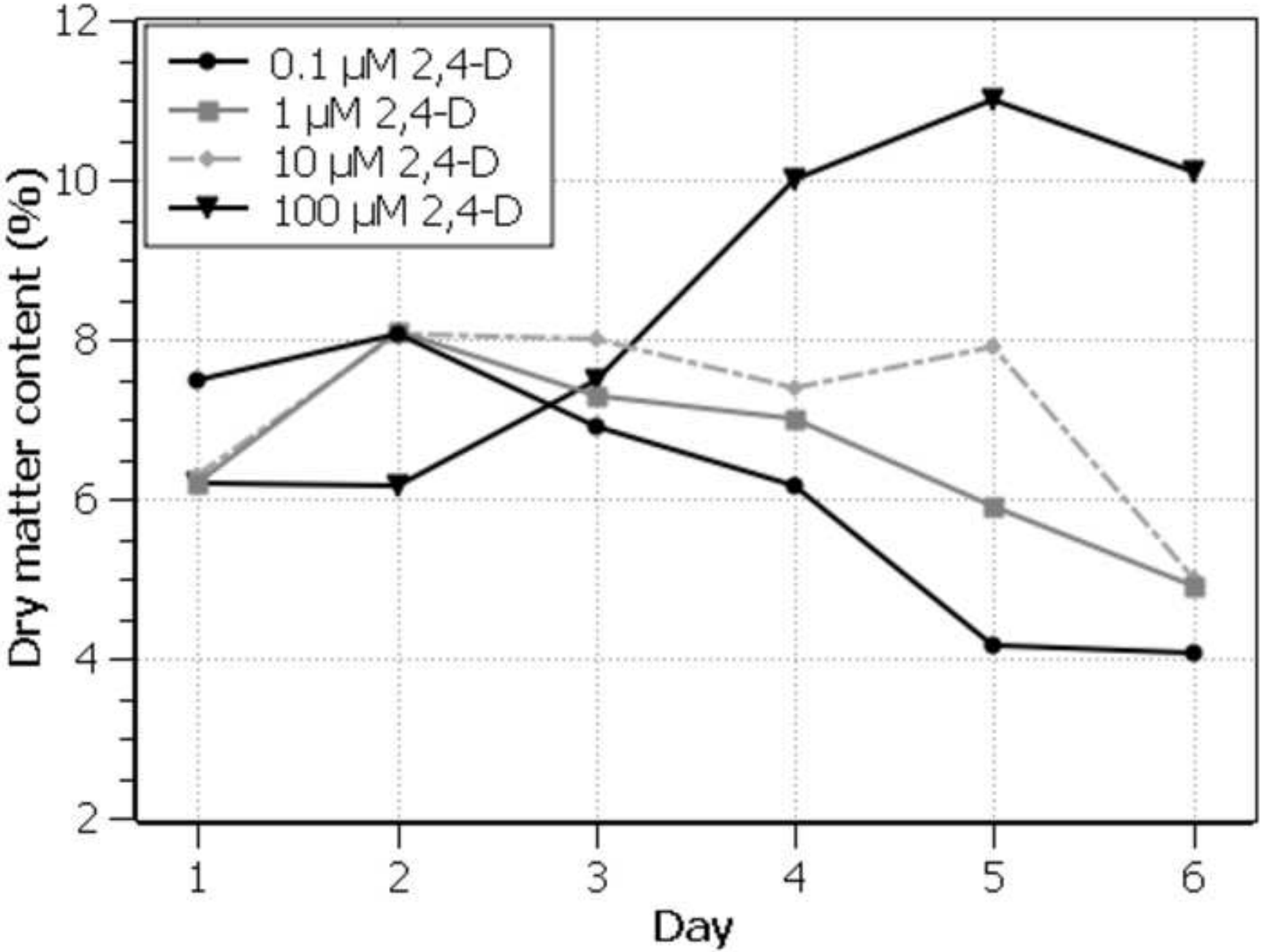

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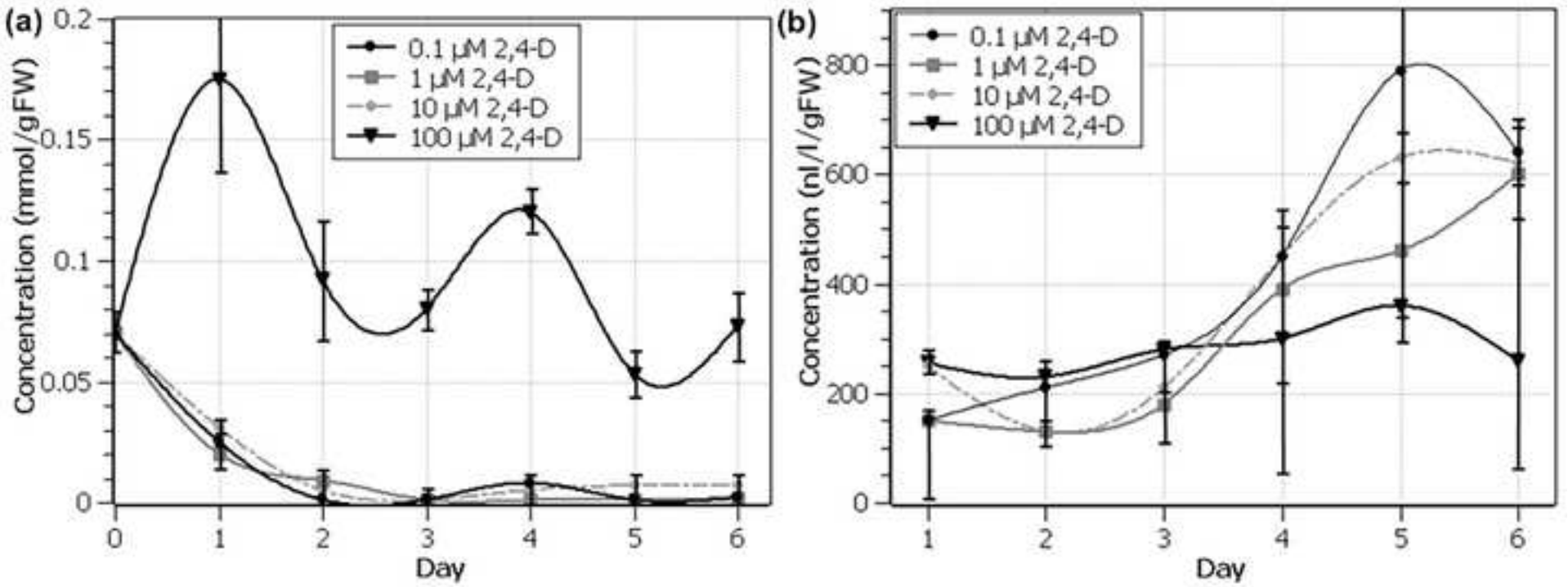

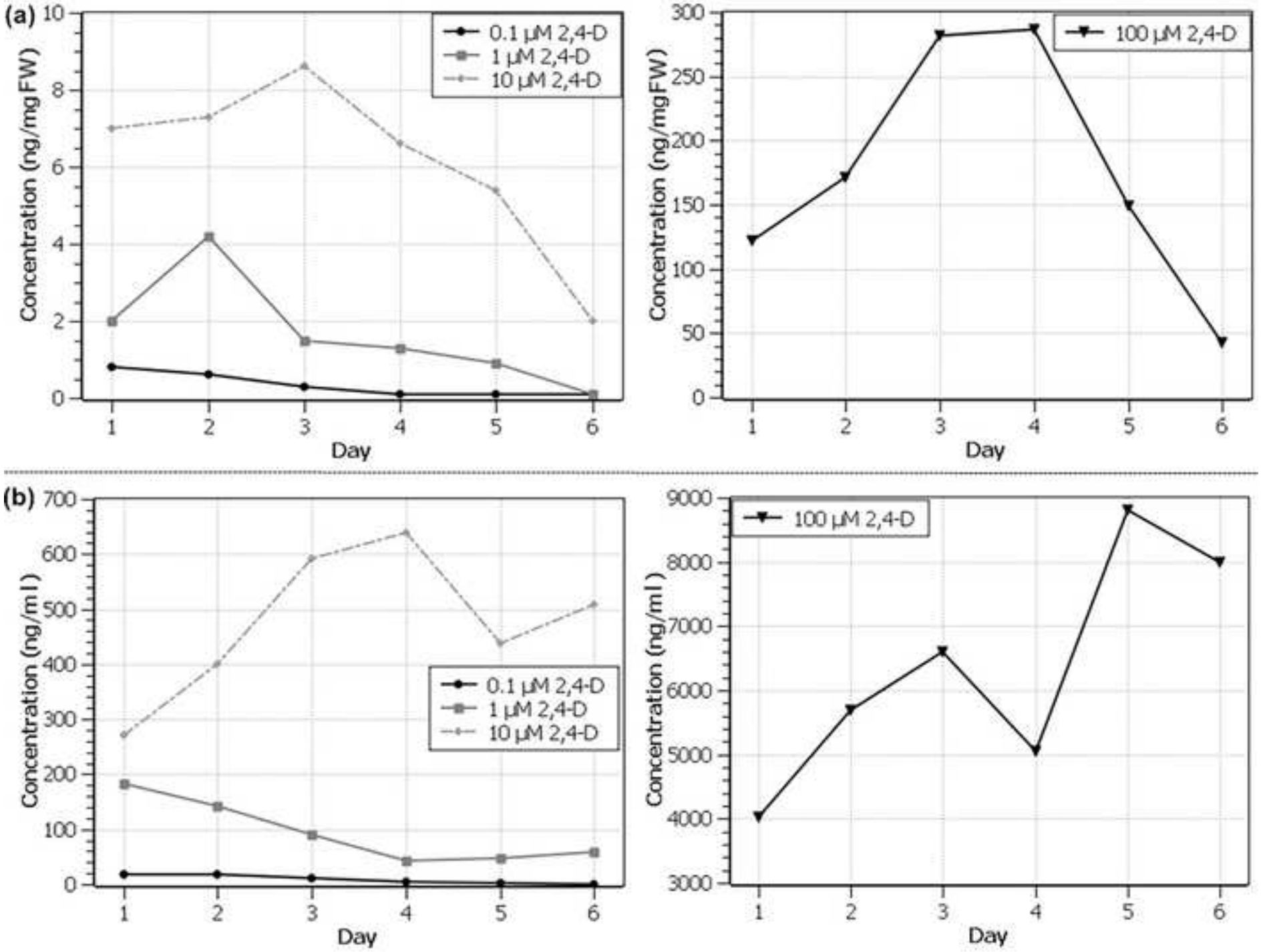

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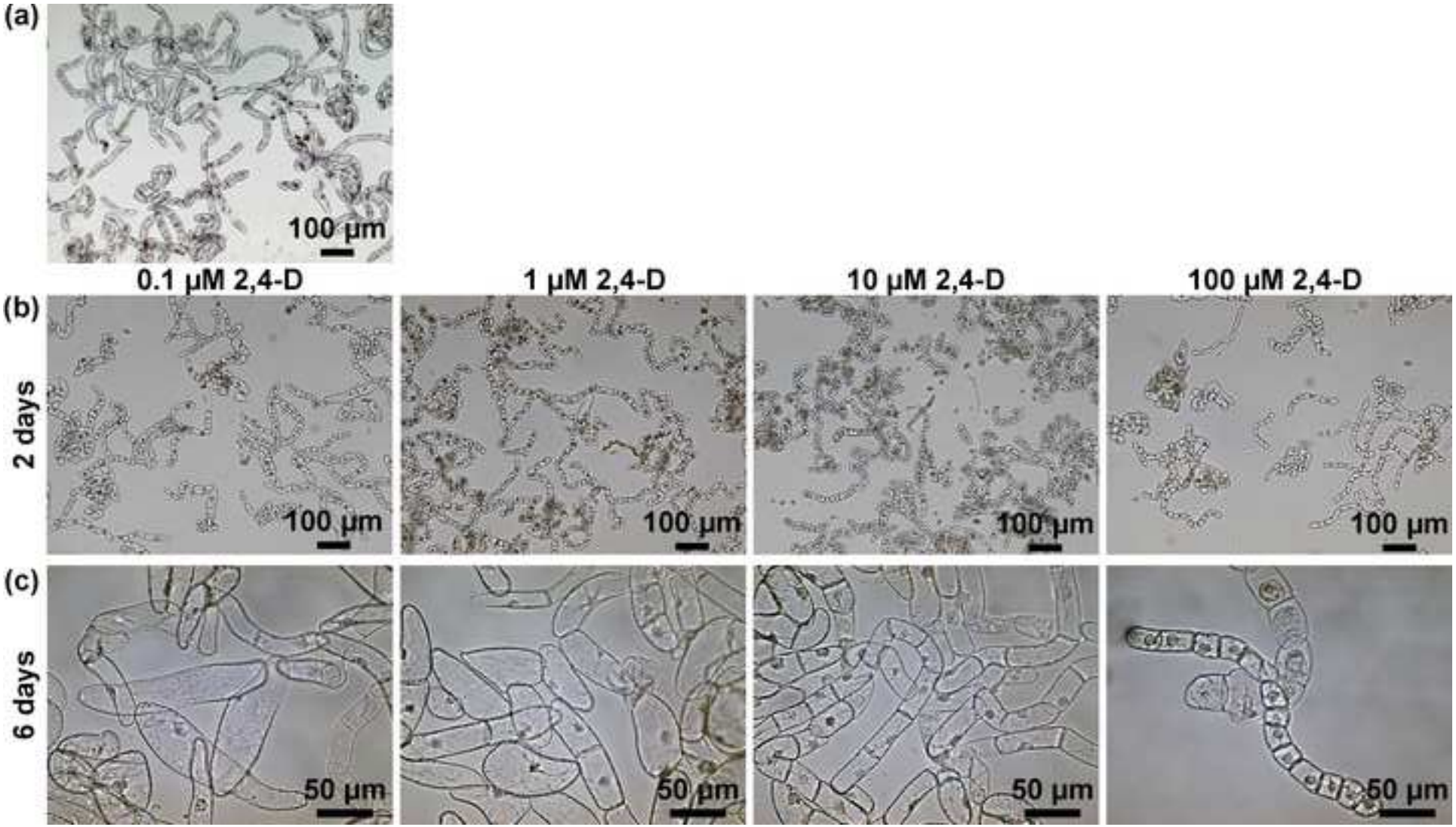

Graphical Abstract