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Droplet-based microfluidics platform for investigation of protoplast development of three exemplary plant species

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Microfluidic technologies offer powerful tools for miniaturized and highly controlled biological experiments, yet their application in plant research remains underexploited. In this study, we present a droplet-based microfluidic platform tailored for the encapsulation and cultivation of plant protoplasts, enabling long-term observation of cell development at nearly single-cell resolution. Protoplasts isolated from leaves of *Nicotiana tabacum*, *Brassica juncea*, and *Kalanchoe daigremontiana* were used to evaluate the platform's suitability across diverse plant species. Our results demonstrate species-dependent responses to microfluidic cultivation, with tobacco protoplasts showing the highest viability. The system permits dynamic tracking of cell fate within individual droplets and supports the quantification of stochastic and concentration-dependent responses to chemical stimuli. Using tobacco protoplasts, we further investigated the effect of low concentrations of cytokinins (BAP) and auxins (NAA) for the early protoplast culture, up to the first division. Low concentrations (20–80 $\mu\text{g}\cdot\text{L}^{-1}$) significantly enhanced cell survival and cell growth, while higher doses did not yield additional benefits. This work underscores the potential of droplet-based microfluidics as a high-resolution, low-volume platform for protoplast-based assays and dose-response screening, with applications across diverse plant biotechnology studies.

Droplet-based microfluidics has emerged as a powerful tool for miniaturized, high-throughput biological experimentation, enabling precise control over cellular environments, single-cell resolution, and massively parallel assays. By compartmentalizing reactions or individual cells into nanoliter-sized droplets, these platforms facilitate highly reproducible, scalable studies that would be difficult or impossible using conventional bulk methods. While this technology has seen wide adoption in biomedical and microbial research^{1,2}, its application in plant biology, particularly for fragile, wall-less cells such as protoplasts, remains limited and does not include prolonged cultivation^{3–5}.

Protoplasts, isolated plant cells without their cell walls, are inherently sensitive and highly responsive to microenvironmental cues. These properties make them both technically challenging and biologically informative as a model system for studying cell viability, division, and differentiation. Beyond their utility in basic research, protoplasts serve as a versatile platform in plant biotechnology, enabling somatic hybridization, transient expression assays, and stable transformation through regeneration into whole plants. They are widely used in DNA delivery and genome editing workflows, providing a direct route to study gene function and engineer new traits without the constraints imposed by cell walls^{6–8}. Traditionally, protoplast research has relied on static culture systems that offer limited control over spatial and temporal conditions, making it difficult to study heterogeneous responses at the single-cell level or in dynamic conditions^{9–11}.

Here, we present an improved droplet-based microfluidic platform, previously demonstrated for successful application in microspore embryogenesis investigation¹². This system allows for the encapsulation, tracking, and longitudinal observation of individual protoplasts or small cell clusters within discrete aqueous droplets with precise control over droplet volume and chemical composition. Our primary objectives were to (i) assess the suitability of this microfluidic approach for maintaining plant protoplast viability over extended culture periods, (ii) evaluate its capacity to monitor early cell division events at nearly single-cell resolution, and (iii) explore the influence of low growth regulator concentrations on developmental outcomes.

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To address these objectives, we employed protoplasts from three dicotyledonous species selected for their complementary experimental properties. *Brassica juncea* was included due to its straightforward protoplast isolation and initial suitability for feasibility testing. *Nicotiana tabacum*, a well-established model organism in plant cell biology, provided a benchmark for system performance and experimental reproducibility. *Kalanchoe daigremontiana* was chosen for its natural propensity for somatic embryogenesis, offering a biologically relevant context to explore developmental potential in microfluidic culture. By using these species as a test case, we demonstrate the platform's capacity to reveal species-specific sensitivities, growth dynamics, and viability trends, as well as seek to identify technical constraints and optimization strategies for long-term culture. This work not only advances technical capabilities for plant single-cell analysis but also establishes a framework for broader applications in regenerative plant biotechnology and precision phenotyping.

Materials and methods

Plant culture

Plant material used in this study included *Kalanchoe daigremontiana* Raym.-Hamet & H. Perrier plants (original plant was obtained in 2021 from a horticultural source in Ilmenau, Germany and maintained clonally in the TU Ilmenau greenhouse. The species identity was verified morphologically by leaf-morphology diagnostics (abaxial spotting/banding)), *Nicotiana tabacum* cultivar Samsun NIC 422, and *Brassica juncea* CR 2664 seeds obtained from the gene bank of the IPK Gatersleben, Germany. Seeds were surface disinfected by immersion in 70% ethanol for 30 s, followed by treatment with 2% sodium hypochlorite (NaOCl) containing a drop of Tween-20 for 5 min. They were then rinsed three times with sterile distilled water for 5 min each. *K. daigremontiana* plantlets were disinfected using the same NaOCl solution for 10 min, omitting the ethanol step. Following disinfection, seeds and plantlets were transferred to Murashige and Skoog (MS) medium containing 30 g·L⁻¹ sucrose and solidified with 4 g·L⁻¹ gelrite. Germination and cultivation were conducted in a controlled environment at 24–25 °C with a 16 h light/8 h dark photoperiod and a light intensity of approximately 5.0 × 10⁻⁵ mol·m⁻²·s⁻¹. *N. tabacum* plants were subcultured every 3–4 weeks. *B. juncea* plants were freshly sown for each experiment and used at 2–3 weeks of age. *K. daigremontiana* plants were propagated vegetatively every 3 months.

Isolation of protoplasts

For standard droplet cultivation experiments, protoplasts were isolated via enzymatic digestion from the top leaves of *N. tabacum*, *B. juncea* and *K. daigremontiana* plants of various age (*N. tabacum*: 3–4 weeks old, *B. juncea*: 2 weeks old, *K. daigremontiana*: 3 months old) following procedure described by¹³. Briefly, leaves were cut into small pieces on sterile sheets of paper and incubated in preplasmolysis solution for 1 h. The solution was then replaced with 5 mL of enzyme mixture (1.6% cellulase and 0.8% macerozyme in BNE9 solution, previously stirred, centrifuged, and filter sterilised), and samples were incubated for 15–17 h at 27 °C in darkness, followed by an additional 30 min of gentle shaking at room temperature (36 rpm). The digested material was filtered through 100 µm sieves (Greiner Bio-One GmbH) into 13 mL round-bottom centrifuge tubes. An equal volume of 20% sucrose solution (5 mL) was added, mixed gently by inversion, and overlaid with 1.5 mL of washing solution to form two phases. After centrifugation at 760 g for 5 min, protoplasts collected at the interphase were transferred to fresh tubes, washed twice with 5 mL of washing solution (centrifuged at 700 g for 5 min each), and finally resuspended in 2 mL of 8pm7 cultivation medium. Based on cell counts using an Anvajo cell counter, the initial protoplast culture was adjusted to a density of 1.5–4 × 10⁵ cells·mL⁻¹.

To accommodate the distinct experimental requirements, two protoplast isolation protocols were applied: one for cross-species comparison and one optimized for *N. tabacum* in the dose–response experiments. For growth regulators dose–response experiments, which were conducted exclusively with *N. tabacum*, protoplasts were isolated from young leaves using a modified version of the protocol described by¹⁴. The initial disinfection step differed: leaves were halved and incubated in 2% PPM (Plant Preservative Mixture, Gentaur GmbH) for 1–2 h at room temperature with gentle shaking, then rinsed with sterile water. All subsequent steps – including cutting, enzymatic digestion (using 0.25% cellulase and 0.25% macerozyme in F-PIN solution), filtration, two-phase separation, and washing – were performed as described above, with minor adjustments: digestion was carried out for 14 h at 26 °C in darkness, followed by 30 min of shaking at room temperature. Isolated protoplasts were resuspended in 4 mL of F-PCN medium without growth regulators at a concentration of 1.6 × 10⁵ cells·mL⁻¹. For establishing a concentration range of hormonal treatments, F-PCN medium containing 4 mg·L⁻¹ BAP and NAA was prepared and serially diluted as needed. The composition of all media used during protoplast isolation and culture is listed in *Supplementary Table S1*.

Microfluidic setup for cultivation in microdroplets

The experimental setup was based on previously described microfluidic systems^{12,15}, with several modifications (Fig. 1). Droplets were generated using a 6-port manifold, with flow rates precisely controlled by a multi-syringe pump (NEMESYS, Cetoni GmbH, Germany). For the standard droplet generation system, 1 mL glass syringes (SETonic GmbH, Germany) were used to deliver the cultivation medium and any effector solutions, while a 2.5 mL glass syringe was used for the carrier phase (PP9). All elements of the system were connected through fluorinated ethylene propylene (FEP) tubing with an inner diameter (ID) of 0.5 mm and outer diameter (OD) of 1.6 mm. This configuration ensured precise flow control.

To minimize mechanical stress on the protoplasts, cell suspensions were not directly injected. Instead, PP9 was introduced into a glass container holding the protoplast suspension, displacing the contents via a smaller polytetrafluoroethylene (PTFE) tube (ID 0.3 mm) toward the droplet generator. The remaining two syringes were used to deliver cell culture medium (for adjusting cell density) and, when applicable, specific effectors. Standard flow rates were approximately 20 µL·min⁻¹ for the aqueous phase and 30 µL·min⁻¹ for the continuous PP9 phase. Following droplet formation (typically 120–300 nL per droplet), they were directed into PTFE incubation tubing

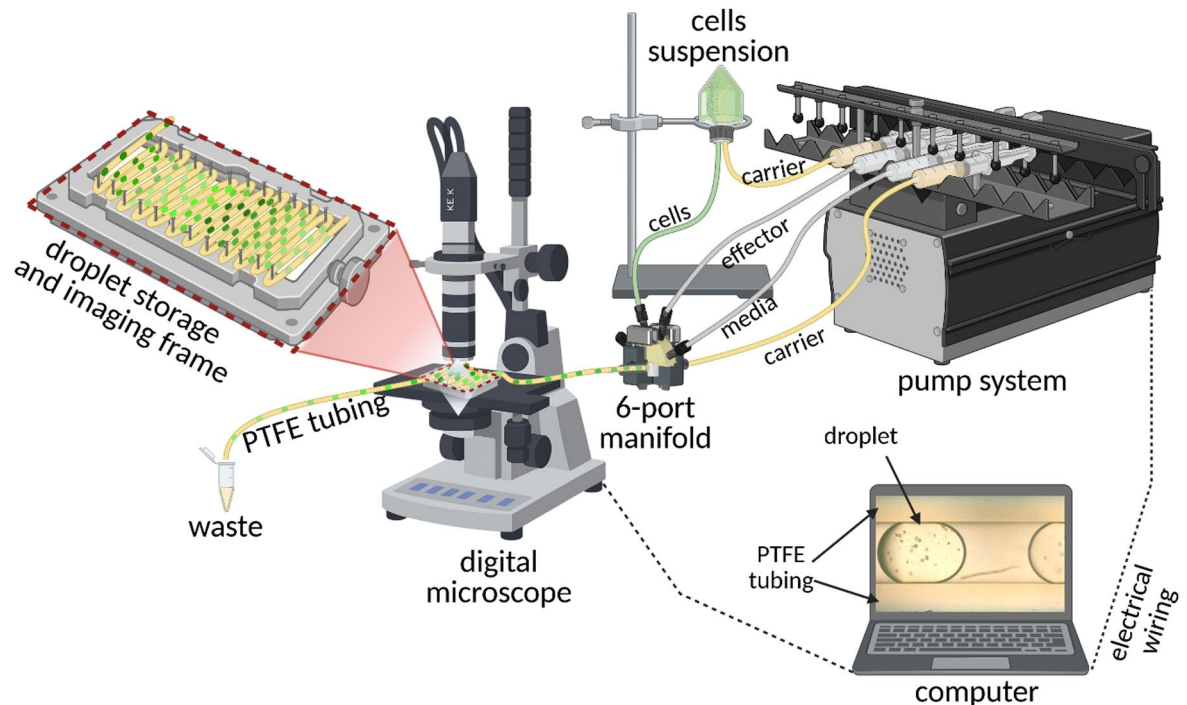


Fig. 1. Experimental microfluidic setup. Droplets were generated via a 6-port manifold allowing delivery of up to two effectors. Protoplasts were supplied indirectly from a suspension container. All flows were controlled by a syringe pump, and droplet formation was monitored by digital microscope. Cultures were incubated in sealed tubing on a frame for imaging. Illustration created with BioRender.com.

with thinner walls (ID 0.5 mm, OD 1.0 mm) to improve air perfusion. After formation, droplets were incubated in the sealed tubes in darkness at 24 °C.

To assess and ensure comparability between experiments, the initial protoplast suspension used for droplet generation was adjusted to $1.5\text{--}4 \times 10^5$ cells·mL⁻¹ (standard droplet cultivation) or 1.6×10^5 cells·mL⁻¹ (dose response experiments). In the droplet-based system, precise control of cell density is inherently limited because the generation flow rate is relatively slow ($20 \mu\text{L} \cdot \text{min}^{-1}$ for the aqueous phase and $30 \mu\text{L} \cdot \text{min}^{-1}$ for the continuous phase), and the suspension was not continuously stirred to avoid generation of additional shear stress, leading to small fluctuations in the number of encapsulated cells. Image-based estimation of actual cell densities within droplets yielded values between 1.06×10^5 and 2.7×10^5 cells·mL⁻¹, confirming that the variation remained within a narrow and biologically comparable range. For reference Petri dish cultures, a lower initial cell density ($1\text{--}2 \times 10^4$ cells·mL⁻¹) was applied to minimize cell aggregation and to better mimic the effective single-cell separation achieved in the droplet system.

Reference cultivation in petri dishes

In parallel with droplet-based experiments, bulk protoplast cultures were prepared as controls under identical conditions. Freshly isolated protoplasts were transferred into 6 cm Petri dishes containing 4 mL of respective cultivation medium (8pm7 or F-PCN) or into 12-well plates with 1 mL of medium per well, supplemented with varying concentrations of the tested effectors. Cell density was adjusted and maintained in the range of $1\text{--}2 \times 10^4$ cells·mL⁻¹.

Growth characterization and data processing

Protoplast cultures were monitored at regular intervals (every 1–3 days) using a digital microscope (Keyence VHX-5000) to assess cell morphology and detect the onset of cell division. In the dose response screening experiment, respective droplets could be found back based on their initial position in the incubation tubing. For quantitative analysis, the number and diameter of cells within individual droplets were measured manually using ImageJ (Fiji) software.

Statistics and reproducibility

Each experiment was conducted in duplicate, with 5–15 droplets analysed per condition in each run. Statistical analyses were performed in Jamovi (version 2.7.5).

For *K. daigremontiana* protoplast size measurements, differences across cultivation days (0, 2, 5, 7) were assessed using the Kruskal–Wallis test, as normality was violated (Shapiro–Wilk, $p < 0.001$). Post-hoc for the growth regulator dose–response experiment with *N. tabacum* protoplasts, data were first tested for normality using the Shapiro–Wilk test. With only a few isolated deviations, most groups did not significantly depart from a normal distribution. Therefore, repeated measures ANOVA was applied to assess the effects of Time (days 1,

3, 5, 7; within-subject factor), Concentration (0, 20, 50, 80, 150 $\mu\text{g}\cdot\text{L}^{-1}$ NAA + BAP; between-subject factor), and their interaction on protoplast growth dynamics. Normality of residuals was evaluated with the Shapiro–Wilk test, and the Greenhouse–Geisser correction was applied where sphericity was violated. Changes in protoplast number and diameter (expressed relative to day 0) were analyzed accordingly. Estimated marginal means (EMMs) with 95% confidence intervals were calculated to visualize significant effects. Statistical significance was defined as $\alpha = 0.05$.

Results and discussion

Comparison of protoplast development in bulk culture and in droplet culture

To evaluate whether the process of loading and cultivation in microfluidic droplets itself influences protoplast growth and survival, a standard bulk culture on Petri dishes was carried out in parallel with each experimental replicate under identical conditions. As shown in Fig. 2, protoplast types exhibited typical developmental patterns in bulk culture, with initial cell divisions observable at around day 5 to 6. *N. tabacum* protoplasts showed a tendency to form aggregates (Fig. 2c, bottom right). For both *N. tabacum* and *K. daigremontiana* protoplasts, development proceeded similarly in droplets and in bulk culture, with droplet-cultured *N. tabacum* protoplasts displaying slightly larger diameters and a more rounded morphology compared to those in Petri dishes. In contrast, while *B. juncea* protoplasts divided normally in bulk, nearly all cells cultured in droplets were non-viable by day 6 (Fig. 2b), indicating a marked sensitivity to the mechanical stress associated with droplet formation. As this variability among plant species in protoplast behavior was observed during droplet cultivation, it is essential to first address the key limitations of this approach before discussing the detailed progression of cell growth and development. One of the primary concerns in microfluidic systems is the generation of shear stress, which varies depending on channel geometry and flow rates, and can lead to mechanical damage and reduced cell viability^{16,17}. Sensitivity to shear stress differs not only between tissue types – such as leaves and hypocotyls – but also between species, influenced by factors such as genotype and cell wall characteristics¹⁸.

Droplet culture generation and cell fate throughout cultivation

As mentioned in the previous section, the droplet generation process had a notable impact on the development of certain protoplasts. Here, we discuss in detail the behavior and developmental fate of protoplasts derived from the three studied plant species.

B. juncea protoplasts exhibited pronounced stress responses immediately following droplet generation. Many cells displayed cytoplasmic condensation with organelle aggregation, accompanied by a significant number of cellular debris in the surrounding medium. While a few protoplasts remained viable during the initial days of cultivation, by day 5 the majority had become visibly shrunk and non-viable (Fig. 3), indicating poor tolerance to the shear stress during droplet formation.

N. tabacum protoplasts initially showed variability during isolation, but the final suspensions used for droplet loading were relatively uniform in morphology and free of large debris. These protoplasts tolerated the droplet generation process well, showing high integrity and minimal signs of mechanical damage or early cytotoxicity. Within the first three days of cultivation, the cells began to aggregate – particularly near interfaces – possibly as a protective response to the microenvironmental factors such as nutrient gradients or surface tension (Fig. 4a–c). This clustering complicated quantitative assessments at the later time points. Nevertheless, by day 6, numerous viable cells had undergone their first division, and microcallus formation was observed (Fig. 4d), suggesting successful adaptation and developmental progression within the droplet-based culture. Our results match the reports of durability of *N. tabacum* leaf protoplasts in microfluidic-based approaches^{19,20}.

K. daigremontiana protoplasts exhibited pronounced heterogeneity in both size and internal composition immediately following isolation. Some cells exhibited intense violet pigmentation due to the accumulation of anthocyanins, while others contained only chloroplasts or were devoid of visible pigments. Post-isolation, these protoplasts had a tendency to aggregate and float in the standard cultivation medium, which hindered uniform dispersion in the loading reservoir and resulted in uneven distribution across droplet segments. Despite this, the majority of cells appeared intact immediately after droplet loading, with minimal debris present. By day 2, distinct cell enlargement was evident in a subset of protoplasts, while others remained static or displayed signs of cell death. By day 5, evidence of cell division was observed in some cells, whereas others continued to increase in size without undergoing division (Fig. 5). This divergent behavior suggests differences in developmental competence across the population, possibly influenced by the original tissue origin.

Importantly, despite the initial heterogeneity, *K. daigremontiana* protoplasts within individual droplets were morphologically distinguishable and sufficiently separated to allow accurate identification, counting, and measurement of cell diameter over time. Based on these analyses, the size distribution of *K. daigremontiana* protoplasts during cultivation was quantified (Fig. 6). Immediately after cell encapsulation, the average protoplast diameter in droplets was approximately 35 μm (range: 15–68 μm ; $n = 235$), compared to 46 μm in parallel Petri dish cultures ($n = 50$). After two days, the droplet-cultured cells reached an average diameter of 49 μm (up to ~100 μm ; $n = 289$), whereas Petri dish-cultured cells averaged 62 μm ($n = 50$). Peak growth in droplets was observed on day 5, with a maximum diameter of 167 μm and an average of 69 μm ($n = 311$), compared to 81 μm in Petri dishes ($n = 50$). Statistical analysis confirmed significant size differences across cultivation days (Kruskal–Wallis, $\chi^2(3) = 191$, $p < 0.001$), with post-hoc Dunn's tests showing that all pairwise comparisons were significant ($p < 0.001$) except between day 5 and day 7 ($p = 1.000$). From day 5 to day 7, droplet-cultured protoplasts maintained a stable size distribution (average 68 μm ; maximum 156 μm ; $n = 304$), similar to the stabilization observed in Petri dishes (average 80 μm on day 7; $n = 50$).

Although only a limited number of cell divisions were observed, making it difficult to perform robust quantitative analysis of mitotic events, the continued increase in size of many protoplasts indicates ongoing metabolic activity and viability. Moreover, the observed variability in the fate of certain protoplast subpopulations

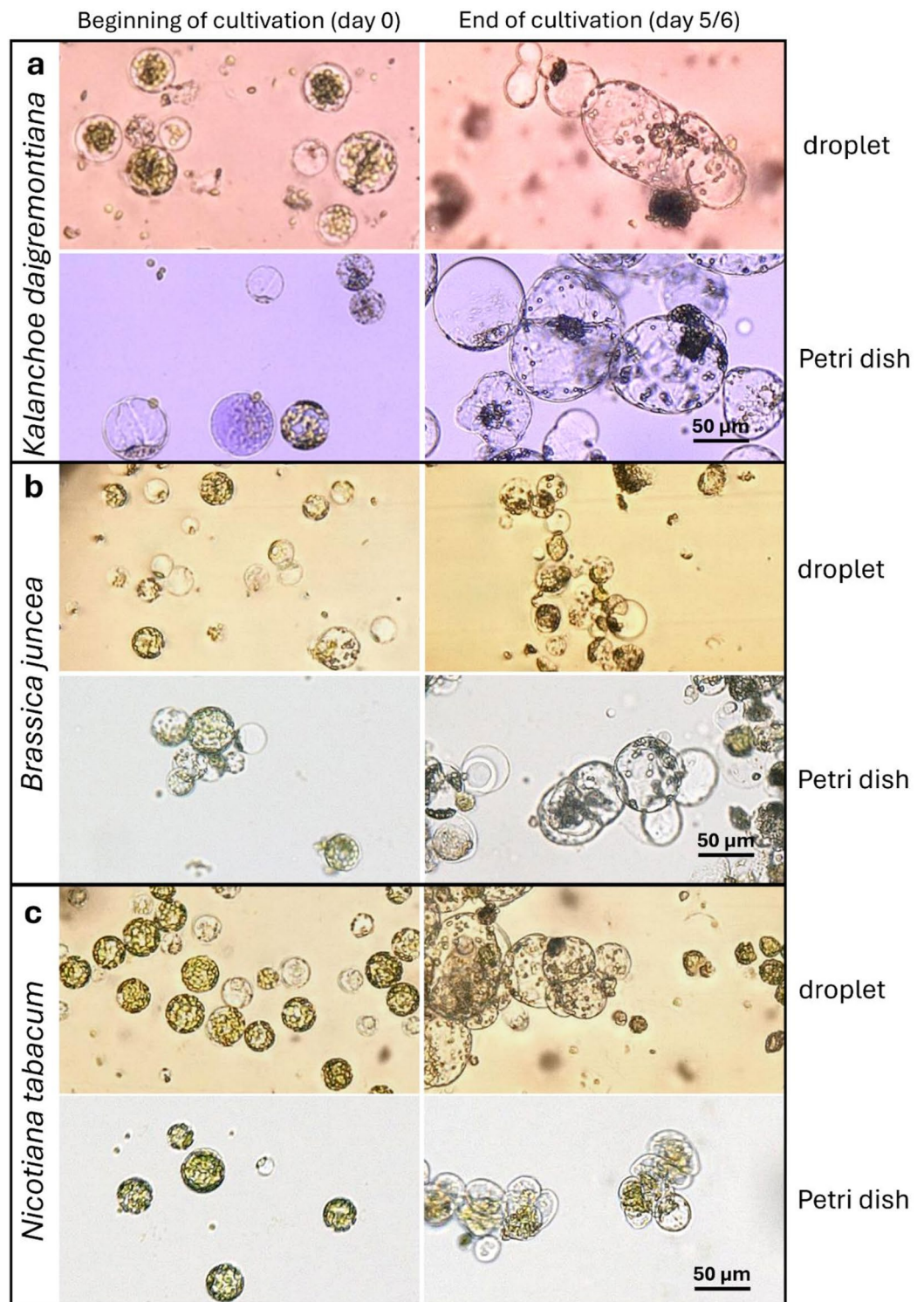


Fig. 2. Comparison of protoplast appearance and development in droplets (top images in each panel) and in Petri dish bulk culture (bottom images), from culture initiation to the expected timing of first cell divisions (around day 5–6): *Kalanchoe daigremontiana* (a); *Brassica juncea* (b); *Nicotiana tabacum* (c).

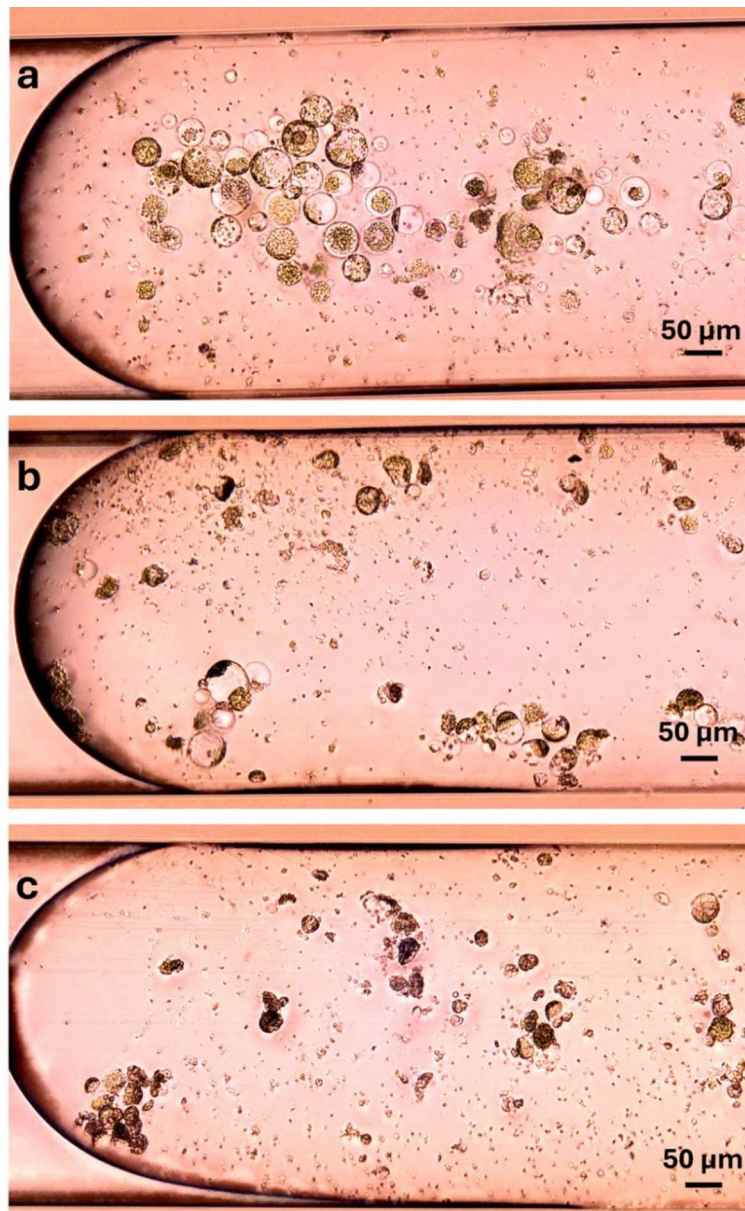


Fig. 3. *Brassica juncea* leaf protoplasts at different time points during droplet cultivation: day 0 – intact protoplasts were introduced into droplets (a); day 1 – some protoplasts maintained integrity, while many exhibited shrinkage and collapse (b); day 5 – nearly all protoplasts had died. Images represent different droplets at each time point (c).

is likely related to the differing regenerative potential of topophysical zones within the leaf²¹, as in our study whole leaves were used as the source material for protoplast isolation. These findings suggest that selecting tissue from zones with higher regenerative capacity could improve the overall outcome of droplet-based cultivation.

Effect of low doses of growth regulators on protoplast survival during the first days of cultivation

Following successful cultivation under standard droplet conditions, *N. tabacum* protoplasts were selected for further exploration of the capabilities and advantages of the microfluidic platform. In this extended experiment, both the storage method of the tubes and the data acquisition strategy was modified, enabling tracking of specific droplets over time. This allowed observation of a much smaller and more defined group of protoplasts, in contrast to the broader population analyzed in previous experiments. As a test case, we examined the effects of plant growth regulators – 6-benzylaminopurine (BAP) and 1-naphthaleneacetic acid (NAA) – administered at significantly lower concentrations than the standard $1 \text{ mg}\cdot\text{L}^{-1}$, ranging from 0 to $150 \text{ }\mu\text{g}\cdot\text{L}^{-1}$. The aim was to determine the minimal concentration required to support protoplast viability during the initial days of cultivation. Figure 7 shows representative outcomes of protoplasts exposed to 0, 20, 50, 80, and $150 \text{ }\mu\text{g}\cdot\text{L}^{-1}$ NAA,

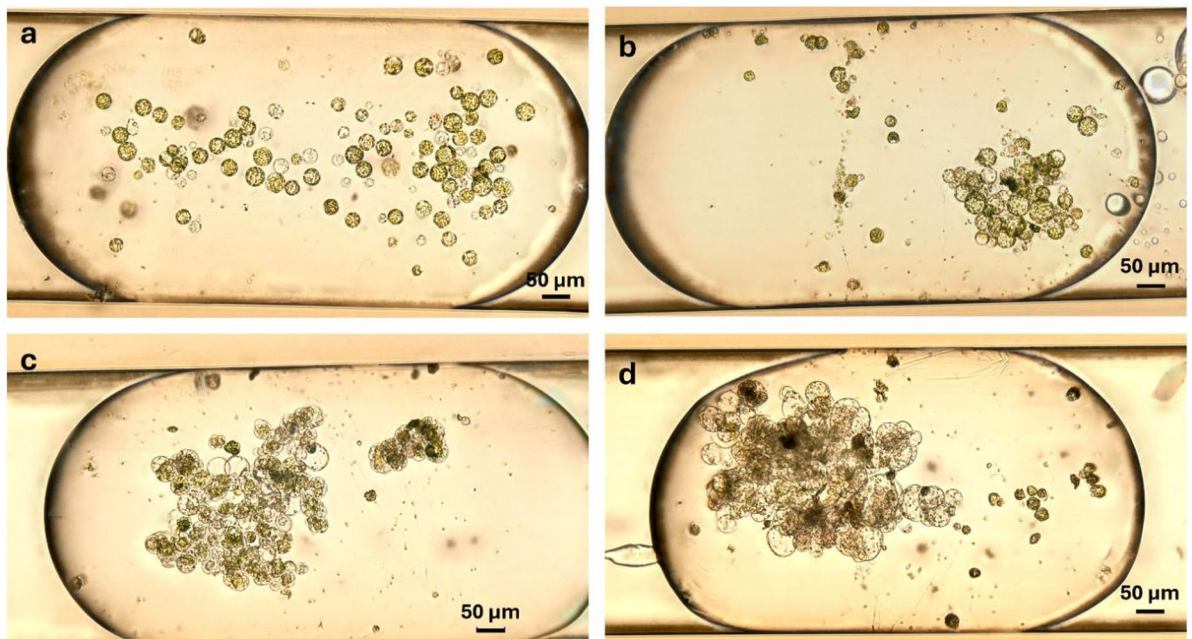


Fig. 4. *Nicotiana tabacum* leaf protoplasts at different time points during droplet cultivation: day 0 – protoplasts encapsulated (a); day 1 – protoplasts remained viable and began contact with each other (b); day 3 – cells aggregated in clusters (c); day 6 – first cell division took place, microcalluses were formed (d). Images represent different droplets at each time point.

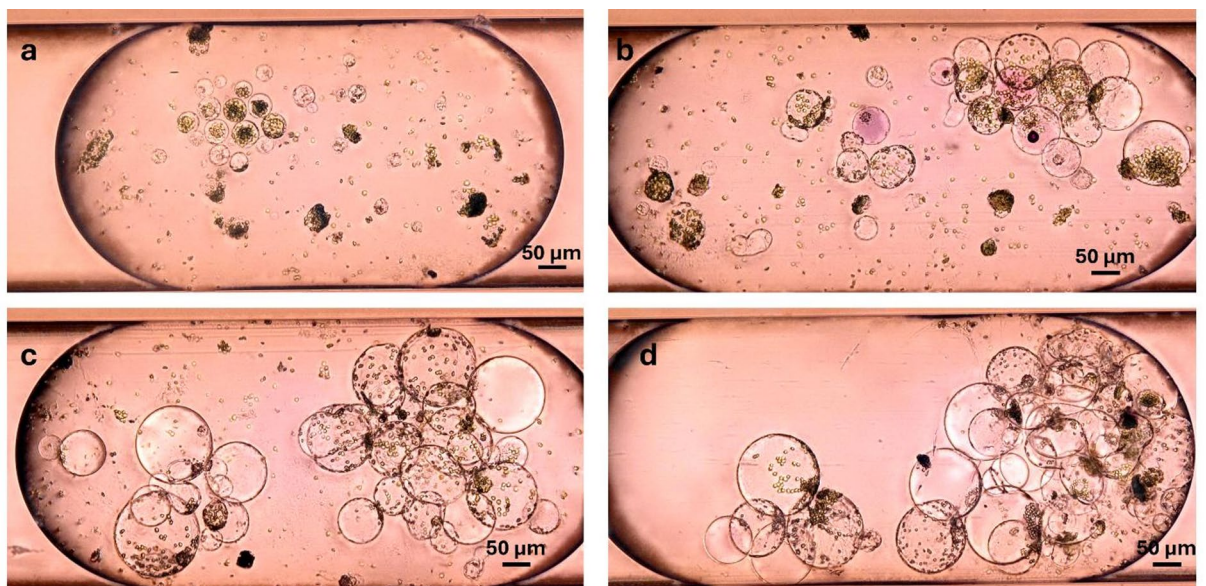


Fig. 5. *Kalanchoe daigremontiana* leaf protoplasts at different time points during droplet cultivation: day 0 – intact protoplasts introduced (a); day 2 – cells increased in size and came into contact with each other (b); day 5 – further enlargement and first cell divisions observed (c); day 7 – morphology remained largely unchanged from day 5 (d). Images represent different droplets at each time point.

with BAP applied at the same corresponding concentrations, as well as standard concentration of NAA and BAP for typical protoplasts culture of $1 \text{ mg} \cdot \text{L}^{-1}$. In the absence of growth regulators, protoplasts rapidly degenerated (Fig. 7a), whereas even low concentrations promoted survival and growth in the early stages (Fig. 7b–d). As reference, no degeneration and clear division of protoplasts by day 7 were observed in the protoplasts cultured in the presence of $1 \text{ mg} \cdot \text{L}^{-1}$ of NAA and BAP (Fig. 7e).

Although the applied growth regulator concentrations were generally insufficient to induce cell division, they did have a clear influence on cell viability. To quantify this effect, we monitored and counted morphologically

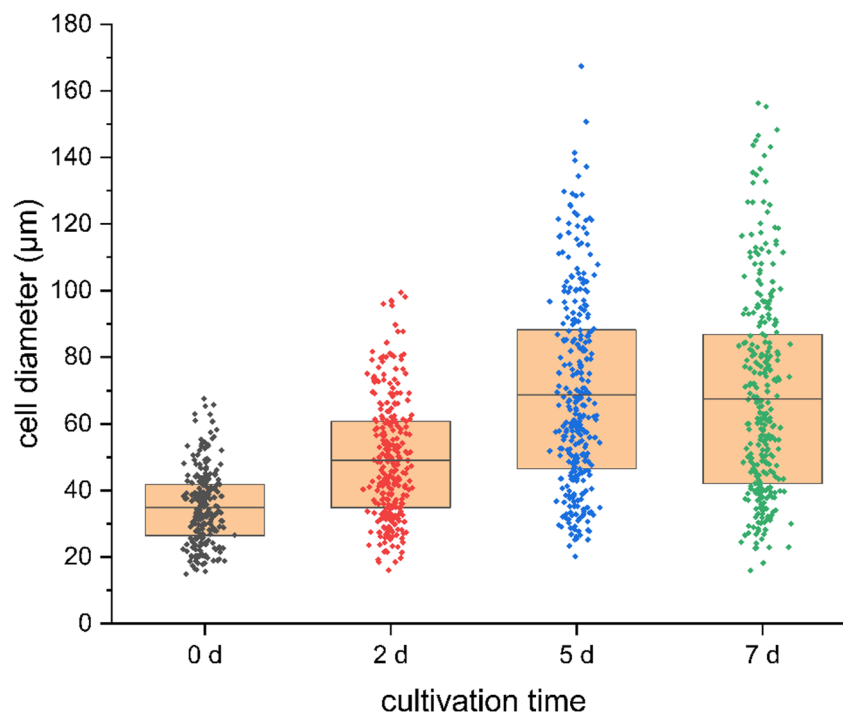


Fig. 6. Size distribution of *Kalanchoe daigremontiana* protoplasts over time during droplet cultivation. Sample sizes (n): day 0–235, day 2–289, day 5–311, day 7–304; measured in 13 droplets for days 0–5 and 12 droplets for day 7. Significant size differences across days (Kruskal–Wallis, $p < 0.001$), with plateau between day 5 and 7.

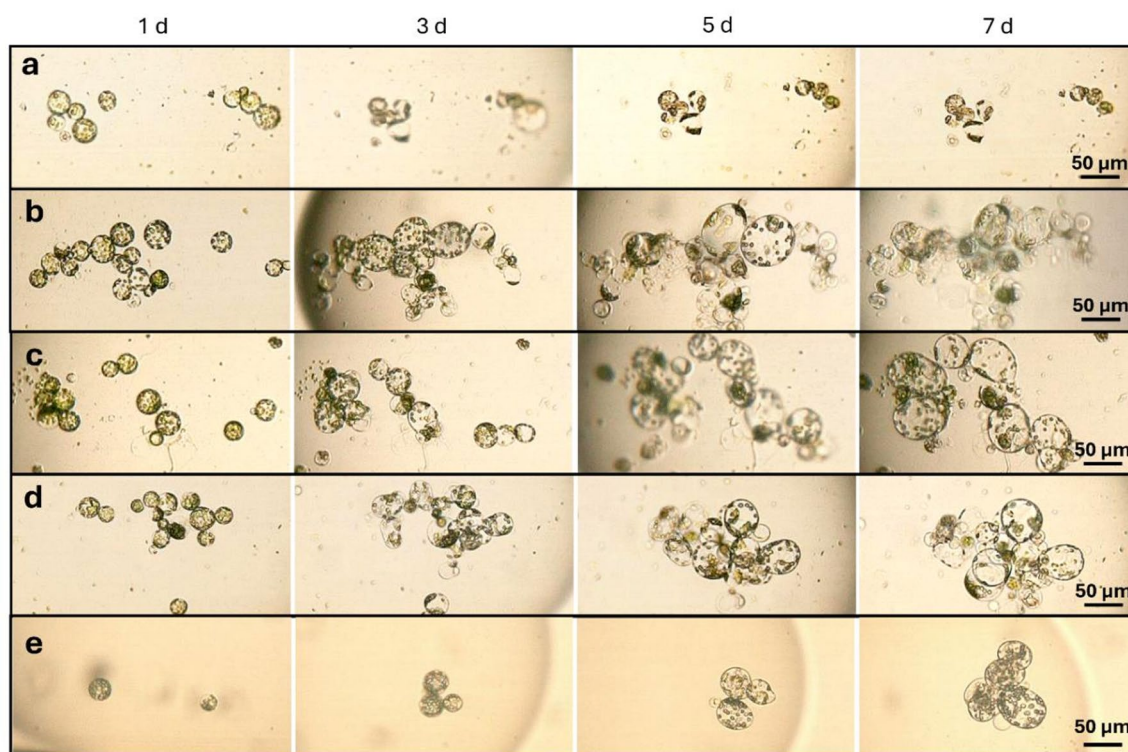


Fig. 7. Development of *Nicotiana tabacum* protoplasts in droplets cultured with different concentrations of BAP and NAA: 0 $\mu\text{g}\cdot\text{L}^{-1}$ (a); 50 $\mu\text{g}\cdot\text{L}^{-1}$ (b); 80 $\mu\text{g}\cdot\text{L}^{-1}$ (c); 150 $\mu\text{g}\cdot\text{L}^{-1}$ (d), 1 $\text{mg}\cdot\text{L}^{-1}$ (e). Protoplasts treated with 1 $\text{mg}\cdot\text{L}^{-1}$ of growth regulators were photographed on days 0, 2, 4 and 7, respectively.

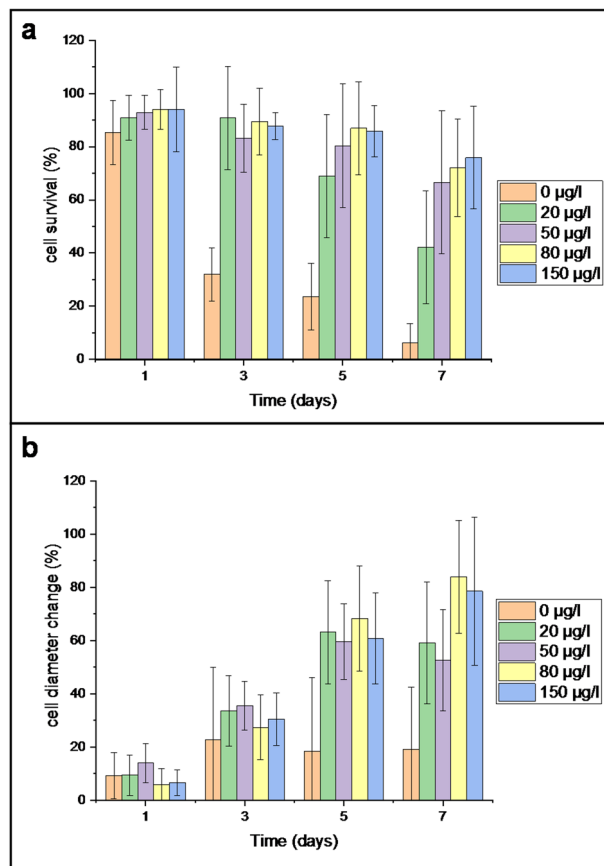


Fig. 8. Characterization of *Nicotiana tabacum* protoplasts during droplet cultivation under varying concentrations of BAP and NAA: Percentage of viable protoplasts over time, relative to day 0 (**a**); Change in average cell diameter during cultivation, normalized to the initial size at droplet loading (**b**). Initial sample sizes (n): 549 in 24 droplets ($0 \mu\text{g}\cdot\text{L}^{-1}$), 512 in 23 droplets ($20 \mu\text{g}\cdot\text{L}^{-1}$), 442 in 17 droplets ($50 \mu\text{g}\cdot\text{L}^{-1}$), 352 in 18 droplets ($80 \mu\text{g}\cdot\text{L}^{-1}$), and 414 in 18 droplets ($150 \mu\text{g}\cdot\text{L}^{-1}$). Error bars indicate standard deviation. Both protoplast number and size were significantly affected by time, concentration, and their interaction (repeated measures ANOVA, $p < 0.001$).

healthy protoplasts in individual droplets throughout the cultivation period, normalizing these counts to the initial number of cells per droplet. Repeated measures ANOVA revealed significant effects of cultivation time ($F(2.53, 244.9) = 33.4$, $p < 0.001$) and growth regulator concentration ($F(4, 97) = 475$, $p < 0.001$) on the relative number of protoplasts, as well as a significant Time $\times$ Concentration interaction ($F(10.1, 244.9) = 132.9$, $p < 0.001$; Greenhouse–Geisser corrected). In the absence of growth regulators, protoplasts displayed a viability of approximately 35% by day 3, which dropped to 20% and 10% on days 5 and 7, respectively. In contrast, treatments with $20\text{--}150 \mu\text{g}\cdot\text{L}^{-1}$ BAP+NAA promoted survival and proliferation, with the strongest responses observed from day 5 onwards at $80\text{--}150 \mu\text{g}\cdot\text{L}^{-1}$, where cell numbers were maintained or increased relative to day 0 (Fig. 8a).

In addition to viability, we assessed changes in protoplast size over time. Repeated measures ANOVA also indicated significant effects of cultivation time ($F(2.26, 191.9) = 237.2$, $p < 0.001$) and concentration ($F(4, 85) = 10.0$, $p < 0.001$) on relative protoplast size, with a significant Time $\times$ Concentration interaction ($F(9.03, 191.9) = 15.6$, $p < 0.001$; Greenhouse–Geisser corrected). In the absence of phytohormones, surviving cells exhibited limited diameter increases – averaging only a 20% increase by day 3, with little to no further growth thereafter. In contrast, exposure to $20\text{--}150 \mu\text{g}\cdot\text{L}^{-1}$ BAP+NAA induced progressive enlargement, with the most pronounced effect at $80\text{--}150 \mu\text{g}\cdot\text{L}^{-1}$, where diameters nearly doubled relative to day 0 by day 7 (Fig. 8b). Estimated marginal means for each time $\times$ concentration condition are provided in *Supplementary Table S2* (cell number change) and *Supplementary Table S3* (cell size change). Although changes in cell diameter are not a definitive indicator of viability or division potential on their own, they are commonly associated with metabolic activity and early cell cycle re-entry in plant protoplasts. The observed diameter changes therefore suggest that the protoplasts remained metabolically active under these droplet-based microfluidic conditions.

Although typical concentrations of growth regulators used in protoplast cultivation and plant regeneration studies are much higher, often ranging from 1 to $5 \text{ mg}\cdot\text{L}^{-1}$, we chose to investigate lower doses. Excessive concentrations of synthetic auxins and cytokinins are known to increase the risk of somaclonal variation, potentially leading to undesired genetic and phenotypic changes in regenerated plants²². By defining the

minimal concentrations that still support protoplast survival and division, the risk of unwanted variation can be reduced. Additionally, using lower doses contributes to minimizing the environmental footprint of in vitro culture protocols^{23,24}.

Overall, the droplet-based platform demonstrated strong potential for controlled protoplast cultivation and early cellular response studies. Beyond the experimental outcomes, the platform provides several practical advantages over conventional Petri dish-based culture methods. The microdroplet format drastically reduces reagent and sample consumption by miniaturizing culture volumes to the nanoliter scale, resulting in significantly lower costs per experiment. In contrast to Petri dishes, which require large media volumes and manual handling, the closed droplet compartments prevent evaporation, minimize contamination risk, and enable precise environmental control. Moreover, compartmentalization allows true single-cell analysis, where individual cells can be monitored independently, providing statistically rich, high-resolution datasets for dose-response and developmental studies.

In addition, the system architecture is highly compatible with process automation, facilitating reproducible handling, parallel experiments, and real-time monitoring. These features make the platform particularly advantageous for high-throughput screenings and parameter optimization studies that are difficult to realize in macroscopic culture systems.

Despite these advantages, the approach has some limitations. Protoplast isolation remains species-dependent and can affect cell viability before encapsulation. The droplet segments can only be moved to a limited extent, as excessive motion induces internal laminar flow and shear stress that may harm sensitive plant cells. The system also requires specialized microfluidic instrumentation and trained operators, which could limit immediate accessibility for some laboratories. Furthermore, nutrient depletion and waste accumulation in static droplets can restrict long-term culture unless media-exchange strategies are implemented²⁵.

Conclusion

In this study, we demonstrated the feasibility of using a droplet-based microfluidic platform for the cultivation of plant protoplasts. The platform allowed for cell encapsulation, maintenance, and observation of protoplasts across multiple species. Notably, species-specific differences in response were evident: *N. tabacum* leaf protoplasts showed the highest robustness and developmental potential, while *B. juncea* leaf protoplasts were highly sensitive to encapsulation-related stress and did not survive extended cultivation in droplets.

The microfluidic setup enables precise tracking of individual cells or defined subpopulations over time and allowed detailed investigation of early cellular responses to phytohormones. Supplementation with low concentration of cytokinin (BAP) and auxins (NAA) significantly enhanced early protoplast survival and growth, without additional benefit observed at higher concentration. These results highlight the sensitivity of the system for dose-response analysis.

Together, our findings demonstrate the potential of droplet-based microfluidics for protoplast-based assays and lay the foundation for future applications in plant developmental biology, regenerative research, and synthetic biology.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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PM: experiments conduction. PM, TW, MK, JC: Conceptualization and writing original draft.

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Declarations

Competing interests

The authors declare no competing interests.

Compliance with guidelines and legislation

All experimental research and field studies on plants, including the collection of plant material, were conducted in accordance with relevant institutional, national, and international guidelines and legislation. No endangered or protected species were involved in this study.

Additional information

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