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Growth Dynamics and Cell Viability in Tomato Suspension Cultures Derived from Different Types of Calli

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Abstract
To establish a dynamic and fine suspension culture, four different methods of tomato cell suspension culture were compared. Hypocotyl explants of the tomato cultivar Jina were used for callus induction on Murashige and Skoog (MS) Medium supplemented with three different phytohormone combinations. Then, one gram of each type of calli was transferred to 50 mL of liquid MS medium with four combinations of auxins and cytokinins to produce cell suspensions. The growth rate, judged by cell turbidity, cell fresh weight, and cell viability was evaluated. The best suspension culture was obtained by using friable calli formed on MS medium containing 1 mg L^{-1} NAA and 0.1 mg L^{-1} kinetin, transferred to the liquid MS supplemented with 2 mg L^{-1} NAA, 0.2 mg L^{-1} 2, 4-D and 0.2 mg L^{-1} zeatin.

Keywords: Calli, cell viability, packed cell volume, phytohormones, triphenyltetrazolium chloride.

Abbreviations: BAP: 6-Benzylaminopurine, CIM: Callus induction medium, MS: Murashige and Skoog, NAA: 1-Naphthaleneacetic acid, OD: Optical density, PCV: Packed cell volume, SCM: Suspension culture medium, TTC: Triphenyltetrazolium chloride, 2, 4-D: 2, 4-Dichlorophenoxyacetic acid.

Introduction

Plant cell suspension cultures, homogenous dedifferentiated cells or cell clusters, are important and versatile *in vitro* systems for molecular and biochemical studies at the cellular level, over-production of natural products, expression of recombinant proteins, *in vitro* mutagenesis, genetic engineering, and other applications. This system provides the possibility to bypass the whole plant complexity and investigate molecular and physiological responses at the cellular level with higher precision and resolution (Moscatiello et al., 2013; Sello et al., 2017).

Important advantages of plant suspension cultures such as large scale application capability, simplicity of production, short cell cycle, independence from environmental conditions such as photoperiod and soil quality, controlled and sterile conditions, high biosecurity, and ease of purification of products, such as recombinant proteins and secondary metabolites, make them excellent tools for laboratory studies (Huang and McDonald, 2012; Rademacher et al., 2019). Plant

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suspension cultures are also used as chemical factories for production of secondary metabolites, recombinant proteins, and biosynthesis of nanoparticles (Fischer et al., 1999; Hellwig et al., 2004; Kieran et al., 1997; Osibe and Aoyagi, 2019). An interesting application of suspension cultures is to use them as a source for protoplast isolation for genome editing experiments. This is an emerging technique for producing transgene-free genome edited crops (Lin et al., 2018).

Since fast-growing and totipotent cell suspension cultures significantly increase the efficiency of subsequent applications such as transformation of CRISPR-Cas9 vectors and regeneration of genome-edited plants (Ondzighi-Assoume et al., 2019; Permyakova et al., 2019), it is necessary to optimize the suspension system for the plant species of interest. Plant suspension cultures are normally initiated by dropping a small piece of friable callus in a liquid plant growth medium, and shake it continuously till a fine suspension cell culture develops (Mustafa et al., 2011). The type of calli used for initiation, nutrient supply, and the type and concentration of phytohormones used for cell suspension establishment have significant impact on the growth dynamics and viability of the cell suspension. Therefore, to establish a fast-growing and fine suspension culture, the optimum conditions need to be determined.

Several different methods have been reported for initiation of suspension culture in tomato *(Solanum lycopersicum* L.) (Ishibashi et al., 2007; Kwon et al., 2003; Nover et al., 1982; Patil et al., 2003; Tewes et al., 1984). Although the methods are similar in terms of using combinations of an auxin (2, 4-D or NAA) and a cytokinin (kinetin or BAP) to develop cell cultures, different molar ratios of the phytohormones, different types of callus, and different varieties or even species of tomato were used in these studies. To establish a long-term tomato suspension culture in our laboratory for subsequent genome editing experiments, we compared four different reported methods for initiation and maintenance of suspension culture under the same conditions and on the same tomato cultivar. We monitored the growth rate, cell morphology, and viability in the different suspensions and introduced the best method.

Materials and Methods

Seed germination

Seeds of tomato, *Solanum lycopersicum* L. cultivar Jina (Ergon Seed, the Netherlands), were surface-sterilized by washing in 96% ethanol for 1 min, 2.5% (v/v) sodium hypochlorite for 5 min, and finally rinsing three times (5 min each) in sterile distilled water. The sterilized seeds were germinated on ½ MS medium (Murashige and Skoog, 1962), solidified with 0.8% (w/v) agar in glass jars at 25 ± 2 °C under 16/8 (light/dark) photoperiod.

Callus induction from in vitro grown plant-explants

Hypocotyl segments, about 5 mm in length, of 21-days old seedlings were used as explants for callus induction on semi-solid MS media with three different combinations of phytohormones: 10 mg L^{-1} NAA callus induction medium 1 (CIM1) (Lin et al., 2018), 2.0 mg L^{-1} NAA and 0.5 mg L^{-1} BAP (CIM2) (Patil et al., 2003), and 1 mg L^{-1} NAA and 0.1 mg L^{-1} kinetin (CIM3) (Venkatachalam et al., 2000). The media were consisted of MS basal salts, 100 mg L^{-1} *myo*-inositol, 2 mg L^{-1} glycine, 0.5 mg L^{-1} pyridoxine-HCl, 0.5 mg L^{-1} nicotinic acid, 0.1 mg L^{-1} thiamine-HCl, 3% sucrose, and 0.8% (w/v) agar. The pH of the media was adjusted to 5.8, and after autoclaving 25 mL of the media were dispensed into each of sterile Petri dishes. Incubation conditions were the same as described for seed germination. After two weeks the explants were transferred to fresh media, and a week later callogenesis percentage, color, morphology and fresh weight of calli were recorded.

Initiation of cell suspension cultures

Fast-growing calli with green to creamy white colors were used for starting cell suspension cultures. Four different MS liquid media, containing four different combinations of phytohormones, were used for cell suspension culture initiation.

The calli formed on CIM1 were transferred to suspension culture medium 1 (SCM1) (Lin et al., 2018), those formed on CIM2 were transferred into liquid medium SCM2 and SCM3 (Patil et al., 2003), and those formed on CIM3 were incubated into liquid medium SCM4 (Ishibashi et al., 2007). The pH of all the media was adjusted to 5.8 before autoclaving. For each treatment one gram of calli was transferred to 50 mL of each liquid medium in 250 mL Erlenmeyer flasks. The flasks were incubated on a rotary shaker at 120 RPM under the similar conditions as that of seed germination.

One-week-old cultures were sieved through a nylon mesh (400 μm pore size) to remove large cell clumps. Then 2 mL of packed cell volume (PCV) of fine-loose aggregates was diluted in 8 mL fresh medium and transferred to 40 mL fresh medium. This procedure was repeated every seven days.

Growth dynamics of cell suspension cultures

In order to compare the growth dynamics of cell suspension cultures, PCV, fresh weight, optical density (OD), and cell numbers of cultures were measured during seven days after sub-culturing from 4-month-old cultures. For all the measurements two replicates were considered. To measure PCV, 10 mL of each sample was transferred to 15-mL tubes and centrifuged at 200 g for 5 min. Then the volume of packed cells was measured as the percentage of total volume (10 mL) (Robledo-Paz et al., 2006).

To measure fresh weight of the suspensions, one mL of each culture was transferred to pre-weighed 1.5 mL tubes and centrifuged at 200 g for 5 min. After discarding the supernatant completely, the tubes were weighed again to deduce fresh weight of the cells (Moscatiello et al., 2013). To measure OD of the cultures, 1-mL aliquots were taken from well-shaked cultures and the OD was measured using spectrophotometer (UV-2100 UV/VIS) at 578 nm wavelength. The number of cells in cultures was counted using a Neubauer hemocytometer. In brief, the chamber was filled by 10 µL of each suspension culture diluted in distilled water (1:1 ratio) and the number of cells was counted under a light microscope.

Determination of cells viability by TTC assay

Number of live cells in different cultures was measured at days 1, 4 and 7 after sub-culturing from a 4-month-old culture according to standard methods, by using Triphenyltetrazolium chloride (TTC). One mL of each sample was centrifuged for 1 min at 9500 g, and the cells were washed twice by 50 mM phosphate buffer, pH 7.5. TTC (Cat#34072, BDH Chemicals Ltd, UK) was dissolved in the same buffer to a final concentration of 2.5 mM, and 1 mL of TTC solution was added to the cells. Samples were incubated for 18 h at 25℃ in the dark without shaking. After the incubation the samples were centrifuged for one min at 9500 g, and the pelleted cells were washed with distilled water. The cell viability was measured by two different methods. The number of cells showing the red color was counted using hemocytometer (Robledo-Paz et al., 2006), and also the amount of red color was measured by spectrophotometer (Castro-Concha et al., 2006; Towill and Mazur, 1975). The red formazan salt (which is produced by biologically active cells) was extracted by 1.5 mL of 50% methanol containing 1% sodium dodecyl sulfate (SDS), at 60 °C for 30 min. The reactions were centrifuged at 1875 g for 5 min and the supernatant was collected. Absorbance of the extracts was measured at 485 nm by using the spectrophotometer.

Statistical analyses

All the experiments and measurements were done in two replicates, except callus

induction experiment, which was performed in five replications. Data were subjected to analysis of variance (ANOVA), and comparison of means was carried out by Tukey HSD test at 5% significance level using R package. The graphs were drawn by using Microsoft Excel software.

Results

To set up an optimized cell suspension culture in tomato, we compared different available protocols on tomato cultivar Jina. To have a comprehensive comparison, we measured different traits of callus and suspension cultures, including callus morphology and size, callus induction rate, and number of the cells, turbidity, and viability in the suspension cultures.

Callus induction rate, callus morphology and size

The rate of callus induction, which was measured as the percentage of explants that formed callus, was different on the tested media ($F_{2,12}$ = 179, P-value = 1.16e-09). The lowest rate was measured on CIM2 with 61.4% compared with 85.3% and 89.5% callus formation rate on CIM1 and CIM3, respectively (Fig. 1A). We also measured root formation rate, which was different on various media ($F_{2,12}$ = 669.4, P-value = 4.91e-13). While no roots were observed on CIM1, 38.6% and 10.5% of calli on CIM2 and CIM3, respectively, produced roots (Fig. 1A).

The size of the calli, judged by the mean of the fresh weight of the calli on each medium, was also different ($F_{2,12}$ = 758.5, P-value = 2.34e-13). Calli on CIM1 were very small with an average fresh weight of 204 mg, while the calli on CIM3 were larger with average fresh weight of 433 mg (Fig. 1B).

The morphology of the calli on different callus induction media was different. The calli on CIM1 were jelly and transparent without any roots or other organs, while calli formed on CIM2 and CIM3 were green or light yellow, fast growing and friable (Fig. 2).

Growth dynamics in cell suspensions cultures

From the three types of calli, cell suspension cultures were started on four different liquid media (Table 1).

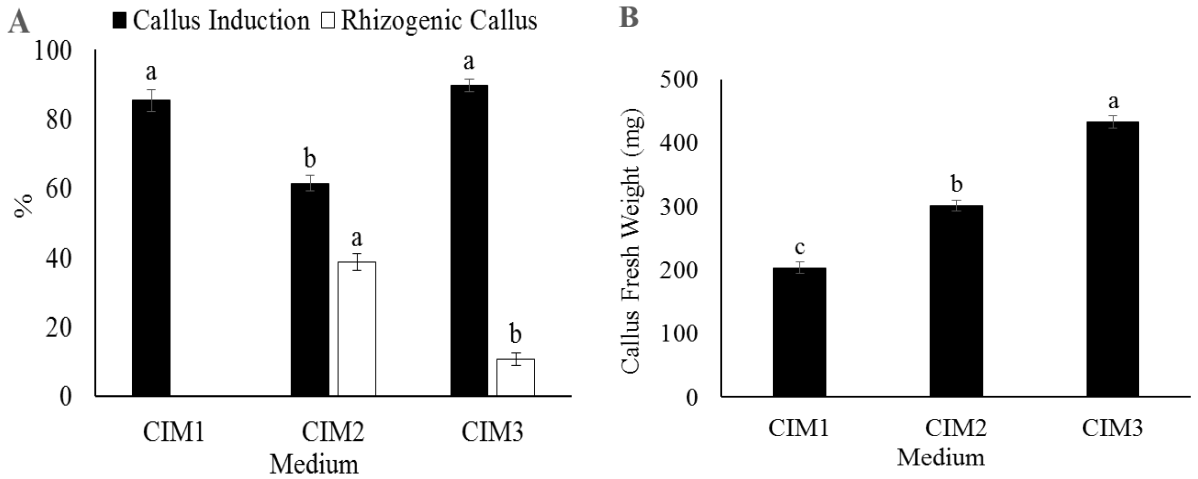

Fig. 1. Effects of different media on callus induction rate and callus characteristics. A. Percentage of callus induction and percentage of rhizogenic calli formed on three callus induction media CIM1, CIM2, and CIM3. B. Callus fresh weight on the three media.

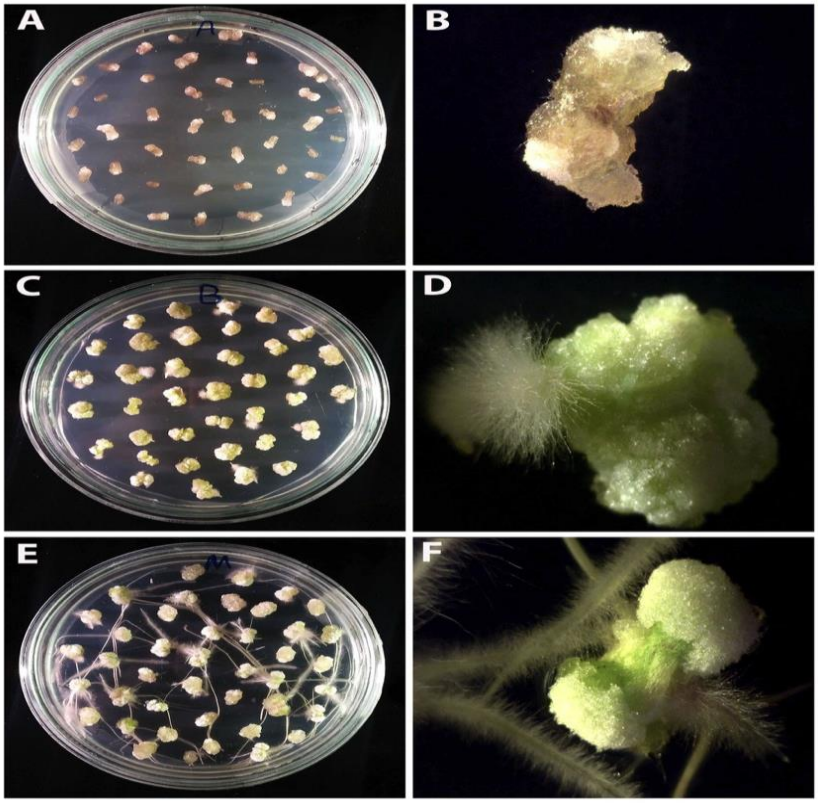

Fig. 2. Morphology of calli formed on three callus induction media. While jelly and transparent calli were formed on CIM1 (A and B), the calli formed on CIM3 were light green/yellow friable (C and D). The Calli on CIM2 were similar to the ones formed on CIM3 in color but they produced more roots (E and F).

Table 1. MS medium with different phytohomrones used in this study for different purposes.

Purpose	Medium	Phytohormones	Reference
Callus induction	CIM1	10 mg L^{-1} NAA (Sigma, N0640, CAS# 86-87-3)	(Lin et al., 2018)
	CIM2	2 mg L^{-1} NAA + 0.5 mg L^{-1} BAP (Sigma, B3408, CAS# 1214-39-7)	(Patil et al., 2003)
	CIM3	1 mg L^{-1} NAA + 0.1 mg L^{-1} kinetin (Sigma, K0753, CAS# 525-79-1)	(Venkatachalam et al., 2000)
Suspension culture	SCM1	1 mg L^{-1} 2, 4-D (Sigma, D6679, CAS # 7084-86-8)	(Lin et al., 2018)
	SCM2	2 mg L^{-1} 2, 4-D + 0.25 mg L^{-1} kinetin	(Patil et al., 2003)
	SCM3	2 mg L^{-1} NAA + 0.5 mg L^{-1} BAP	(Patil et al., 2003)
	SCM4	2 mg L^{-1} NAA + 0.2 mg L^{-1} 2, 4-D + 0.2 mg L^{-1} Zeatin (Sigma, Z0164, CAS# 13114-27-7)	(Ishibashi et al., 2007)

The growth dynamics of the suspension culture was measured in a 7-day time course after sub-culturing based on fresh weight of cells, absorbance at 578 nm (OD_{578}), number of cells, and PCV.

A similar trend was observed in all the measured traits in all the media. There was a lag phase till day 4 and then the exponential phase started. SCM4 showed the highest values for all the measured factors (Fig. 3A-D), suggesting that the growth rate in this medium was higher compared to the other media.

Since the trend in the different traits was very similar, we calculated the correlation between the traits. We observed a high correlation between the different measured traits ($r>0.97$).

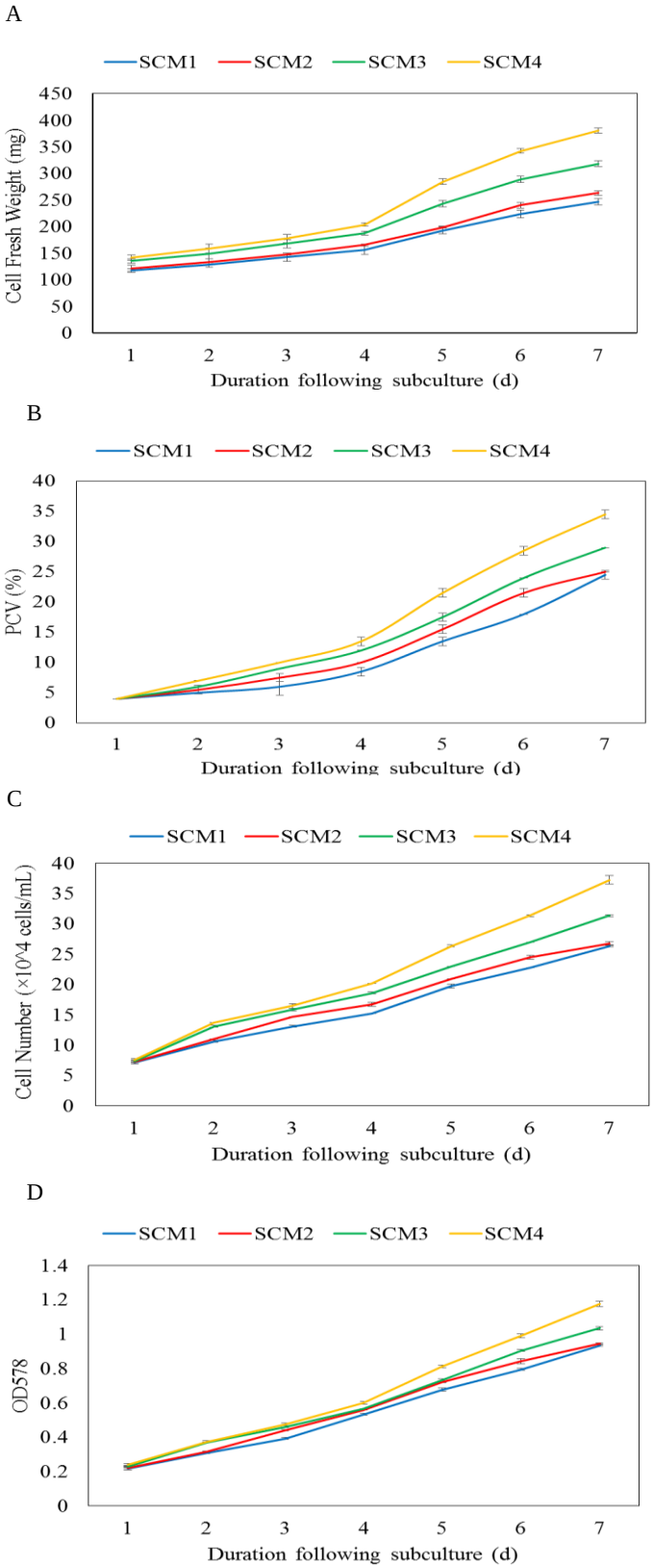

Fig. 3. Growth dynamics of the four different suspension cultures. The cell fresh weight (A), PCV (B), cell number (C), and OD_{578} (D) of 4-month-old suspension cultures were measured from day 1 to 7 after sub-culturing. For all the measured factors SCM4 showed the highest values.

Morphology and viability of the cells in suspension cultures

Plant cell suspension cultures are normally a composition of single cells, cluster of cells, and thread-like structures. The ideal suspension culture is the one with mostly clusters of 5-10 active and fast dividing cells with less vacuolated and long cells (Mustafa et al., 2011; Williams et al., 1988). We compared the cell composition in different media under the microscope to compare the morphology of the cells, and performed the TTC test to compare the viability of the cells in different media.

The cell suspensions in SCM1 and SCM2 were similar in shape and viability. There were usually large single oval-shaped cells (Fig. 4A and C) with no clusters or threads of small cells. The viability test, judged by the lack of red color in TTC assay, revealed that most of these cells are not biologically active (Fig. 4B and D). Although the large single cells were also observed in SCM3 and SCM4, clusters and threads of single small cells were more frequent in those media (Fig. 4E and G). Most of the small cells showed the red color after TTC assay, suggesting that they are very active cells (Fig. 4F and H). In particular the number of cell clusters in SCM4 was higher than that in SCM3.

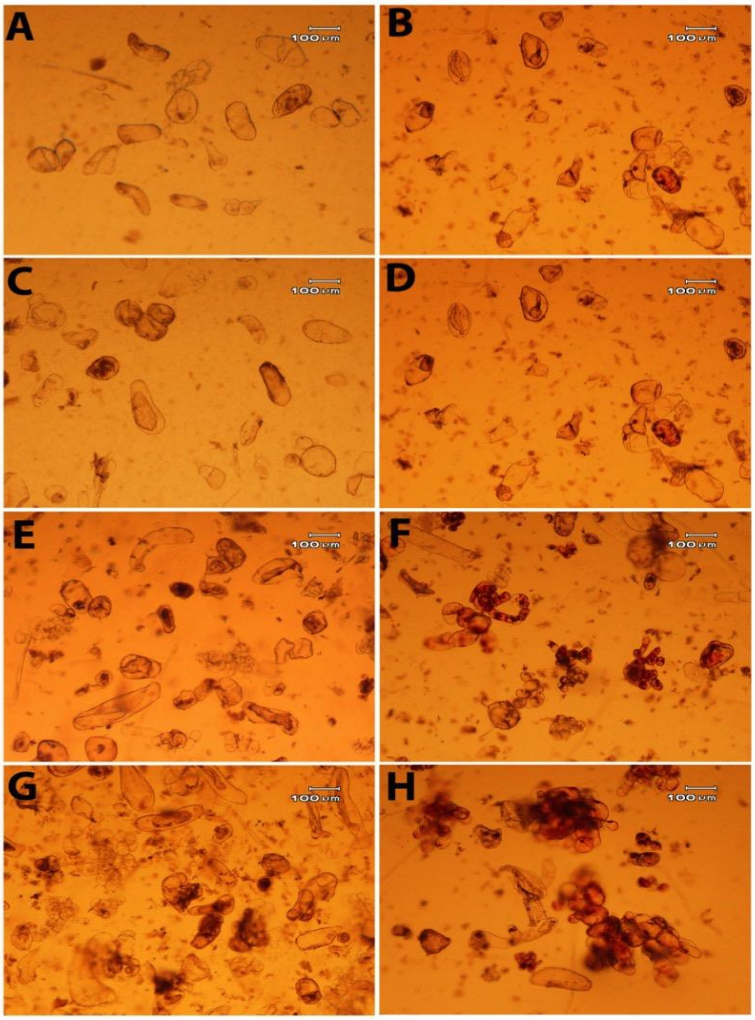

Fig. 4. Cells morphology and viability in different suspension media. The suspension in SCM1 and SCM2 mostly consisted of single vacuolated cells (A and C), with low viability (B and D). Cells in SCM3 and SCM4 were smaller in size and formed aggregates of 5- 10 cells (E and G) and were mostly biologically active (F and H). The viability is judged by the density of red color after TTC assay.

TTC is reduced by living cells to Formazan, an insoluble red precipitates, because of mitochondrial activity. The cells viability is quantified either by counting red cells under microscope (Robledo-Paz et al., 2006) or by measuring optical density of the TTC reaction in 485 nm (Castro-Concha et al., 2006; Towill and Mazur, 1975). Here we measured the cell viability in different media at 1, 4, and 7 days after sub-culturing by using the both methods. The results obtained from the two methods were very consistent ($r \geq 0.86$); showing that in all the media the viability of the cells increases till day 4 and then it decreases (Fig. 5A and B). However, the decrease in SCM3 and SCM4 was much slower than in the other two media. The OD in medium E decreased by 49.4% from day 4 to day 7; however this decrease was only 2.3% in SCM4.

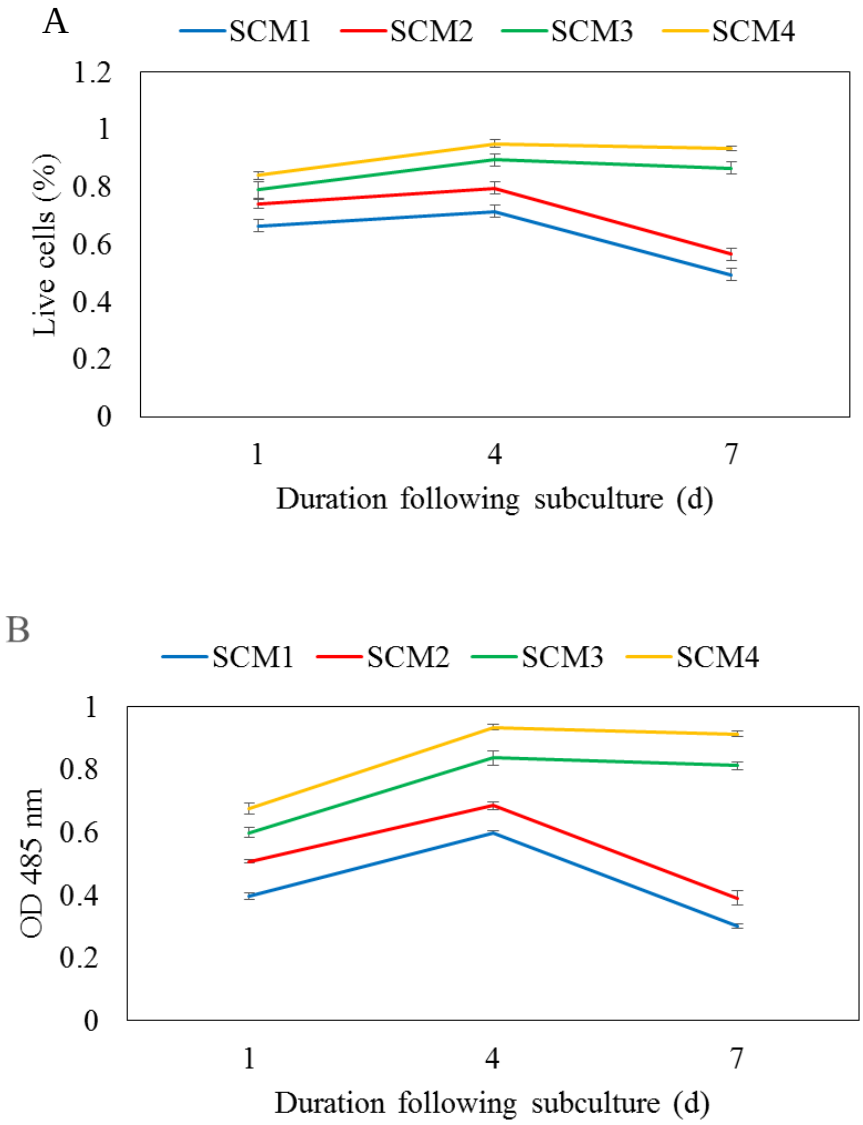

Fig. 5. Quantification of viability of the cell suspension cultures. During 1, 4, and 7 days after sub-culturing, the number of live cells (showing red color) was counted (A), and also the OD_{485} of the red color extracted from the suspension cultures was measured at 485 nm (B). The cell viability drops sharply in SCM1 and SCM2 at 4 days after sub-culturing.

Discussion

Suspension culture systems are promising tools to develop transgene-free genome-edited plants, and therefore developing fine fast-growing suspension cultures, with high plant regeneration rate, in crops like tomato will facilitate fast expanding genome editing methods in this valuable crop. Here we compared different suspension culture methods in tomato.

We used three established protocols for callus induction from hypocotyl explants of tomato. In the presence of high concentration of NAA (10 mg L^{-1}), small, jelly, and transparent calli were formed (Fig. 2A and B) as expected (Lin et al., 2018). Lower concentration of NAA (1 mg L^{-1}), along with kinetin (0.1 mg L^{-1}), resulted in expected light yellow to green calli (Fig. 2C and D) (Ishibashi et al., 2007). The yellow-green colour of the calli can be explained by the protective role of cytokinins, specially kinetin, in minimizing breakdown of chlorophylls and carotenoids (Khokhar and Mukherjee, 2011).

Using NAA with BAP or kinetin also induced rhizogenic calli (Fig. 2E and F). This combination was used for wild tomato species, *S. chilense*, for callus induction (Patil et al., 2003), and that could be a reason for the different response we report here. The response of different cultivars of one plant species to phytohormones, especially auxins and cytokinins, is different (Jehan and Hassanein, 2013; Wayase and Shitole, 2014).

The liquid media containing NAA as the auxin source resulted in fine and viable suspension cultures. In particular SCM4 was superior to other media in terms of cell growth rate, cell morphology, and cell viability. The growth rate and viability of the suspension cultures in the presence of high concentrations of 2, 4-D (1 or 2 mg L^{-1}), were lower than that in the presence of NAA (Fig. 3, 4 and 5). This is probably due to the toxicity effect of 2, 4-D compared to NAA, especially when it is used in suspension culture in direct contact with plant cells (Nover et al., 1982; Tewes et al., 1984). Positive effects of high concentrations of 2, 4-D in inducing fine and fast-growing suspension cultures in *S. lycopersicum* cv. Micro-Tom is reported (Lin et al., 2018), which again shows the genotype-dependent response to phytohormones in tomato.

We analysed the growth dynamics of the suspension cultures by counting the cells, measuring PCV, fresh weight of the cells per mL, and OD of the suspension culture, which are the most reported parameters to evaluate the viability and growth rate of cell suspensions (Neumann et al., 2009). The growth curve was similar to the curves reported for suspension culture in tomato (Li, 2011; Nover et al., 1982; Patil et al., 2003; Tewes et al., 1984) and other plant species including *Arabidopsis thaliana* (Sello et al., 2017), rice (Toriyama and Hinata, 1985), ginger (Tan et al., 2016), and tobacco (Robledo-Paz et al., 2006).

Our results showed that the results of the different methods of measuring the cell suspension culture dynamics in tomato were very consistent with high correlation ($r>0.97$). This is in agreement with previous reports (Tewes et al., 1984). Therefore, we suggest using OD_{578} as the reliable and fast method for monitoring cell growth dynamics in tomato suspension cultures.

Metabolic activity of cells such as reduction of tetrazolium salts to Formazan, and physical attributes of cells such as cytoplasmic streaming and integrity of membrane are used for cell viability assessment in suspension cultures (Castro-Concha et al., 2006; Coder, 2001; Strober, 2015). The TTC assay revealed that the frequency of biologically active cells in those media containing 2, 4-D was low. The morphology of the cell was also different. The cells in SCM1 and SCM2 media were often large, vacuolated, and there were rare cell clusters (Fig. 4A and C; Fig. 5A and B). In contrast, SCM3 and

SCM4 were mostly composed of cell clusters of biologically active small cells (Fig. 4E and F; Fig. 5A and B). The viability test by measuring OD_{485} or counting the red cells resulted in similar results with high correlation ($r>0.85$), therefore we suggest measuring the OD_{485} as the easier method for comparing viability in tomato suspension cultures.

In conclusion, in our experimental conditions, the method reported by Ishibashi and co-workers (Ishibashi et al., 2007) was the best method for suspension culture establishment and maintenance. Factorial experiments aiming to test combinations of different types of calli in different suspension media will help to further optimize tomato suspension culture. Comparison of the regeneration rate of protoplasts isolated from SCM3 and SCM4 suspension cultures is the necessary step to evaluate the efficacy and feasibility of using these cultures for genome editing purposes.

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Conflict of interest

The authors indicate no conflict of interest for this work.

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