Document downloaded from:

http://hdl.handle.net/10251/167604

This paper must be cited as:

Rivas-Sendra, A.; Corral Martínez, P.; Camacho-Fernández, C.; Porcel, R.; Seguí-Simarro, JM. (2020). Effects of growth conditions of donor plants and in vitro culture environment in the viability and the embryogenic response of microspores of different eggplant genotypes. Euphytica. 216(11):1-15. https://doi.org/10.1007/s10681-020-02709-4

The final publication is available at

https://doi.org/10.1007/s10681-020-02709-4

Copyright Springer-Verlag

Additional Information

Effects of growth conditions of donor plants and *in vitro* culture environment in the viability and the embryogenic response of microspores of different eggplant genotypes

Alba Rivas-Sendra[†], Patricia Corral-Martínez[†], Carolina Camacho-Fernández, Rosa Porcel and Jose M. Seguí-Simarro[*]

Cell biology Group - COMAV Institute, Universitat Politècnica de València, Valencia, Spain

[†] These authors contributed equally

* Corresponding author. e-mail: seguisim@btc.upv.es Tel/Fax: +34963879047

Abstract

Notwithstanding the importance of eggplant in global horticulture, doubled haploid production in this species is still far from being efficient. Although acknowledged to have a role in the efficiency of androgenesis induction, factors such as the growth conditions of donor plant or the *in vitro* culture environment have not been deeply explored or not explored at all in eggplant, which leaves room for further improvement. In this work, we investigated the effects of different *in vivo* and *in vitro* parameters on the androgenic performance of different eggplant genotypes, including two hybrids and a DH line. The *in vivo* parameters included the exposure of donor plants to different temperature and light conditions and to increased levels of boron. The *in vitro* parameters included the use of different concentrations of NLN medium components, sucrose and growth regulators, and the suspension of microspores at different densities. Our results showed that whereas greenhouse temperature variations or boron application did not to have a positive influence, greenhouse lighting influenced their viability, thereby conditioning the embryogenic response. Changes in different sucrose, salts and hormone levels had different effects in the genotypes studied, which correlated with their genetic constitution. Finally, we determined the best microspore density, different from that previously proposed. Our work shed light on the role of different factors involved in eggplant microspore cultures, some of them not yet studied, contributing to make microspore culture a more efficient tool in eggplant breeding.

Keywords: androgenesis, growth regulators, microspore culture, microspore embryogenesis, *Solanum melongena* L.

Introduction

In microspore embryogenesis, microspores are switched from microsporogenesis towards an alternative *in vitro* pathway where they develop as haploid or doubled haploid (DH) individuals. After specific *in vitro* stress treatments, some microspores are insensitive to it and follow a gametophytic-like developmental program (Satpute et al. 2005), but other microspores are successfully induced to divide sporophytically, becoming haploid or DH embryos. DH technology is a convenient tool in plant breeding, since it produces fully homozygous plants (pure lines) in a reduced period of time and with fewer resources. It is also useful for fundamental cell and molecular biology research, as well as for plant genetics (Abdollahi et al. 2009; Bueno et al. 2003; Jouannic et al. 2001). Compared to other DH technologies, microspore embryogenesis has the advantage of the thousands of microspores, potentially embryogenic, contained in a single anther. However, a still limited knowledge of the factors involved in the induction of this experimental process precludes a wider use of this technique. The low embryogenic response of many crops still precludes the massive use of DH technology, which can only be successfully exploited in few high responding species and genotypes. Among the different factors involved in the embryogenic response, the genotype is very well known and perhaps the most important one (Dunwell 2010; Seguí-Simarro and Nuez 2008). Although yet poorly studied, culture conditions of the plants used as donors of microspores have also been proposed as having an important role in the eventual response of microspores to embryogenesis (Dunwell 1976; Dunwell 2010). During anther development, the developmental window within which microspores can be deviated towards embryogenesis is also an important parameter. For most species, this window has been stablished around the first pollen mitosis (Seguí-Simarro 2010). Other factors as the type and duration of the stress-based inductive treatment, the composition of the culture medium and the density at which microspores are plated are also parameters to be studied and adjusted in order to optimize this process (Seguí-Simarro et al. 2011).

There are different technical alternatives to generate DHs. Among them, microspore culture is the method of choice in the species where possible, since it is the method where conditions are most controlled, thus providing the highest efficiency. In eggplant, however, the most widely used method is still anther culture (Salas et al. 2011; Salas et al. 2012). Microspore culture in eggplant has still a short history (reviewed in Seguí-Simarro 2016). Miyoshi (1996) was the first to develop a well-documented and working protocol of isolated eggplant microspore culture to induce embryogenesis, produce callus and regenerate DHs. He established the basis of the conditions for induction and the composition of the culture medium used in further studies. Since then, a number

of works have reported on the evaluation of different modifications of the medium to try to improve the efficiency of the technique and to promote progression of the microspore-derived embryo. For example, the addition of polyethylene glycol was proposed to improve the rate of callus induction and its proliferation (Corral-Martínez and Seguí-Simarro 2012). The addition of epibrassinolide was proposed to improve callus induction, although callus proliferation was negatively affected (Corral-Martínez and Seguí-Simarro 2014). The use of abscisic acid was assessed in order to prevent the undifferentiated callus growth observed soon after the formation of globular embryos. However, abscisic acid was not able to completely avoid embryo disorganization (Corral-Martínez and Seguí-Simarro 2014). Gum arabic, a mix of arabinogalactans and arabinogalactan proteins, was the only compound able to promote embryo development beyond the globular stage, but the MDEs formed presented an aberrant morphology, being defective in shoot apices (Corral-Martínez and Seguí-Simarro 2014). In general, the efforts to improve this technique focused on the *in vitro* environment, and while there are still *in vitro* conditions to refine, to date not much attention has been devoted to greenhouse/growth chamber environments to which donor plants are exposed, and to their consequences on the androgenic response.

As seen, the efforts to improve this technique are still limited, and there is still a wide room for improvement in order to understand what factors influence the androgenic response in eggplant, and how to modulate them. In this work we aimed to explore some of these factors in several eggplant genotypes through isolated microspore culture. We focused on some factors relative to donor plant conditions, including the effects that greenhouse temperature and light have in the *in vivo* development of microspores within the anthers of donor plants, and how this affects their further *in vitro* embryogenic response. We also studied the possible effect that foliar applications of boron might have on microspore/pollen viability. Indeed, boron is essential for normal development of reproductive organs, and increased boron levels were found to increase tube growth and germination in areca (Liu et al. 2013), and pollen quality and viability in other solanaceous crops (Peñaloza and Toloza 2018). The response to alterations in several parameters of the *in vitro* culture environment such as microspore density and sucrose, salts and hormone levels was also studied. Together, our results may be useful to make isolated microspore culture a more efficient tool in eggplant breeding.

Materials and methods

Plant material

We used as donor plants 2 eggplant F1 hybrids and a DH line. In this work, we identified the hybrids as E1 and E2, since they were proprietary material protected under confidentiality agreements. The DH36 line is a DH line developed by us (Rivas-Sendra et al. 2017b) from cv. 'Bandera' a commercial hybrid from Seminis. Seeds of all materials were germinated in growth chambers in plastic dishes and transferred to seedling trays with commercial growing substrate and kept at 20ºC with a photoperiod of 16/8 and light intensity of 200 μmol m^{-2} s^{-1}. After one month, seedlings were transplanted to 30 cm pots in a pollinator-free greenhouse at the COMAV greenhouses (campus of Universitat Politècnica de València, Spain, GPS coordinates: 39º29'01" N, 0º20'27'' W). Pots were filled with coco peat and irrigated and fertilized using a drip irrigation system. Plants were grown under natural light in air-conditioned greenhouses where temperature was initially set up to 22ºC but subjected to seasonal fluctuations (22±3ºC). Plants were pruned and trained with vertical strings.

Application of boron to donor plants and evaluation of microspore viability

The experiment consisted of a randomized complete block design with three concentrations of foliar fertilizer of soluble concentrated 15% (w/v) boron (Labifol). Five replicates of each treatment were done, totaling 20 pots (one plant per pot). Three concentrations were made, equivalent to 100, 150, and 200 mg/L. Boron was applied to the leaves on a weekly basis during the treatment (three weeks), at a dose of 0.16 L per plant and application. Control plants were applied the same volume of water. To evaluate microspore viability after boron application, anthers from buds containing a majority of vacuolated microspores and young pollen grains (the suitable stages for microspore embryogenesis) were sliced and washed to extract microspores/pollen. For fluorescein diacetate (FDA) staining (Rivas-Sendra et al. 2017a; Rivas-Sendra et al. 2019), two drops of microspore/pollen suspension were placed on a slide with 5 μl of 1/100 FDA solution, incubated for 10 min and observed under a epifluorescence microscope. Data collected from five different microscope fields were arcsine transformed and subjected to analysis of variance (ANOVA). Post-hoc comparisons with Duncan's multiple range test were used to find out differences between groups ($p \leq 0.05$).

Isolated microspore culture

Before the *in vitro* culture of microspores, for each genotype we determined the anther length containing the appropriate microspore/pollen developmental stage. Microspores were *in vitro* cultured in liquid medium as described in Corral-Martínez and Seguí-Simarro, (2014). Briefly,

anthers at the right stage were dissected, surface disinfected and crushed to release the microspores. Microspores were isolated through 41 µm filters, centrifuged three times, suspended in distilled water, inoculated in plates and incubated at 35°C in darkness during three days. Then, microspores were recovered from cultures, resuspended in NLN medium (Nitsch and Nitsch, 1967) supplemented with 2% sucrose, 0.5 mg/l of 1-naphthaleneacetic acid (NAA) and 0.5 mg/l of 6-benzylaminopurine (BAP), and cultured continuously at 25°C in darkness.

Correlations between temperature and light conditions of donor plants and microspore viability, divisions and callus production

We performed different microspore cultures using several randomly chosen plants from the E1 and E2 genotypes as donors of microspores. In total, 10 cultures of E1 and 6 cultures of E2 were performed, with a minimum of 9 replicates (culture dishes) per culture. In parallel, we recorded the temperature and light of the greenhouse module where these plants were confined, using thermometers and luxometers installed in the greenhouse and connected to a computer to transmit automatically the values every 20 minutes during the entire duration of the experiment (from January to September). We defined light as the illuminance directly received by plants and therefore measured inside the greenhouse, and expressed in kiloluxes (klx) in both cases. For each of the cultures analyzed, we calculated the average temperature and light corresponding to the day when flower buds were excised (from 00:00 up to the moment of excision, approximately at 10:00 hours), the whole day before, and two and seven days before. For each culture, we calculated the viability of microspores at the moment of isolation (day 0) and just after the 3-day heat shock (day 3). For this, we stained them with fluorescein diacetate and observed and counted as described above to calculate the percentage of viable microspores. The percentage of dividing microspores (microspores with two or more nuclei divided by the total number of microspores) was calculated by observing samples of microspore cultures at day 7, stained with DAPI as described in Rivas-Sendra et al. (2019), under the inverted epifluorescence microscope. Paired comparisons to study possible correlations between them were performed calculating their coefficient of determination (R^2), defined as the square of the Pearson coefficient of correlation.

Medium modifications

Different variants were applied to the culture medium. First, changes in concentrations of hormones (100% vs 20%), NLN salts and vitamins (1x vs 2x) and sucrose (2% vs 4%, 5% and 10%) were assayed. Microspore density was initially adjusted to 5×10^5 microspores/mL for all the experiments, unless otherwise specified. In the experiment of different plating densities, we evaluated the

response of cultures with the following plating densities: 5×10^4, 1×10^5, 2×10^5, 3×10^5, 4×10^5, 5×10^5, 1×10^6 and 2×10^6 microspores/mL. 5×10^5 microspores/mL is the density considered as standard in previous works (Corral-Martínez and Seguí-Simarro 2014; Miyoshi 1996; Rivas-Sendra et al. 2015), so we used it as control. For all cases, we adjusted the different densities using a Neubauer improved chamber according to Camacho-Fernández et al. (2018). To assess the influence of different culture conditions, callus-like structures were observed and counted under a stereomicroscope after 30 days of culture. On the one hand, we counted the total of calli present in a plate, in order to estimate the efficiency of induction. On the other hand, we counted the number of calli with a size exceeding 1 mm, in order to estimate their growth. We expressed these two parameters per unit of volume (mL) of culture medium, except for the experiment of different plating densities, where the androgenic response was expressed as the number of calli per 5×10^5 microspores in order to normalize the effect of the different densities used. For each medium modification studied, three different repeats were performed, with at least three replicates (plates) per condition. We performed a test of analysis of variance with a $p\leq0.05$ to identify putative significant differences. Subsequently, we performed a Fisher's least significant difference (LSD) test for multiple comparisons with the aim of combining the treatment effects in homogeneity groups. Differences were considered as significant when the p-value exceeded 0.05.

Results

Effect of temperature and light conditions of donor plants

For this experiment, we used plants of the E1 and E2 hybrids. Due to seasonal fluctuations of temperature, plants were exposed to temperatures of ~20ºC in winter, ~23ºC in spring and ~26ºC in summer (Figure 1A). Anyway, the range of temperature fluctuation (6ºC) was low. The absence of records during two weeks in June was due to a temporary failure in the recording system. Light measured within greenhouses was variable, ranging from 3 to 10 klx, depending on the season. As typical in Mediterranean countries, the weekly average illuminance was minimal in winter, and then increased progressively until June (summer solstice), and then decreased again during autumn months, In August-September, a drastic decrease in average illuminance was due to unstable, cloudy and rainy weather, typical from the Valencian region at the end of summer - beginning of autumn. The viability measures made for E1 and E2 microspores (Figures 1B and 1C, respectively) ranged from 25% to 55% at the moment of isolation for *in vitro* culture and from 10% to 30% after the 3-day heat shock treatment. This treatment was the most likely cause of the viability drop.

For both genotypes, microspore viabilities at day 0 and day 3 were clearly proportional, with maxima during January-May and minima from June to July, the months where average temperature increased ~3ºC and illuminance was the highest. In August and September, however, viabilities increased up to levels comparable to spring months. This coincided with a drop of illuminance due to cloudy weather, as explained above, whereas temperatures remained unchanged. Together, these facts strongly suggested a relationship between the drop in viability of freshly isolated microspores and high illuminance conditions.

To gain a deeper insight on this, for each microspore culture we calculated the average light and temperature to which E1 and E2 donor plants were exposed during the day when flower buds were excised (up to the moment of excision, approximately at 10:00 a.m.), and one, two and seven days before. These data were crossed with viability of cultures at days 0 (at the moment of microspore isolation) and 3 (just after heat shock), number of microspore divisions counted at day seven, and number of calli per dish at day 30. As seen in Tables 1 and 2, the R^2 coefficients revealed that in general, there was no correlation between the parameters studied, or the correlation found was weak. However, there were some cases where R^2 revealed strong correlations ($R^2 \geq 0.90$) between the two variables studied (bold R^2 coefficients in Tables 1, 2). This was the case of light during one and two days before bud excision. We found that for both E1 and E2 genotypes, the luminous flux received by donor plants during the day before and even during the two days before bud excision explained more than 90% of the variability found for the viability levels observed just after microspore isolation. However, R^2 coefficients for the correlations between illuminance at the day of bud excision (from 00:00 h up to the very moment of excision), and 7 days before excision, were very low, near zero, indicating the existence of a null or very low correlation. In other words, microspore viability appeared negatively influenced by the amount of light to which donor plants were exposed during the two days previous to bud excision, but not by the immediate light exposure nor by long-term (7 days) exposure.

In addition to viability at day 0 (microspore isolation), viability at day 3 (just after heat shock exposure) was also negatively affected in the E2 genotype, as revealed by the high R^2 coefficients observed (bolds in Table 2). In the E2 genotype, viabilities at days 0 and 3 were also influenced by temperature, and the number of calli at day 30 was influenced by both temperature and illuminance conditions during the last 7 days before excision. On the contrary, the corresponding R^2 values in E1 revealed a very low correlation in these cases. Together, these results suggested a strong correlation between illuminance and *in vivo* microspore viability in both genotypes. However, correlations between illuminance and day-3 viability, between temperature and viability (at both days), and between temperature/illuminance and number of calli were more genotype-dependent.

Effect of foliar application of boron

We evaluated the potential effect of the addition of boron to donor plants in microspore viability by FDA staining (Figures 2A, B). As seen in Figure 2C, no boron application was found to give rise to viability percentages significantly higher than controls without boron addition. Indeed, the highest concentration clearly produced a detrimental effect in microspore/pollen viability. This neutral or negative effect appeared specific for microspore/pollen viability, or at least not extended to the general development of the plant, since all donor plants presented a good, healthy aspect in general, and also in their different parts (leaves, stems, branches, flowers, etc.) at all the boron concentrations used (Figure 2D). In conclusion, boron did not to have a positive effect on viability of eggplant microspores/pollen, at least at the suitable stage for microspore embryogenesis.

Response to changes in growth regulators, salts, vitamins and sucrose concentrations

Next, we performed microspore cultures to evaluate the effect of different *in vitro* factors. Once vacuolated microspores and young pollen grains were isolated and *in vitro* inoculated (Figure 3A). the first divisions could be observed in some microspores after some days (arrowheads in Figure 3B), whereas others remain arrested. Later on, microspore-derived calli (Figure 3C) were formed by undifferentiated proliferation of the microspore-derived embryos initially formed (Corral-Martínez and Seguí-Simarro 2012). Upon transference to solid medium, shoots regenerated from their surface (Figure 3D). Shoots were then excised and transferred to rooting medium (Figure 3E), where full plantlets regenerated *in vitro* (Figure 3F) three-four months after the onset of microspore culture, being ready for ploidy analysis by flow cytometry. Individuals found to have a 2C DNA content (the same than donor plants and twice the content of haploid individuals) were considered as DHs with no need for confirmation with molecular markers. In microspore cultures, only isolated microspores/pollen are present in culture dishes. Thus, all embryos, calli and plants obtained from these cultures must necessarily come from haploid microspores/pollen, as confirmed by our previous studies with microsatellite molecular markers in plants regenerated from microspore cultures (Corral-Martínez and Seguí-Simarro 2012). Confirmed DH individuals were then ready for *ex vitro* transference and acclimation.

The first assays of embryogenesis induction in microspores of the E1 and E2 genotypes, performed in parallel to the experiments described in the previous section, revealed that microspores of these genotypes had a low ability to undergo cell division and proliferation, yielding ~15-30 calli/mL under standard culture conditions. For this reason, we decided to include in the next experiments the DH36 eggplant DH line which, under the same conditions, produces hundreds of calli. As a first

approach to evaluate the effect of salts, vitamins, sucrose and growth regulators, we first increased the concentration of the NLN medium (including their salts, their vitamins and the sucrose added) and reduced the concentration of growth regulators. The increase in NLN medium and sucrose concentration was found to have no positive effect in E1 microspore cultures neither on the total production of calli (Figure 4A) nor on the number of calli larger than 1 mm (Figure 4B) with respect to controls. A reduction in the concentration of growth regulators to 20% increased 3.4-fold the total number of calli and 3.7-fold the number of calli larger than 1 mm. Combining 2x NLN, 4% sucrose and 20% growth regulators was found positive for the number of calli obtained (a 4.6-fold increase in the total number of calli, but not for the number of calli larger than 1 mm (Figure 4B). With respect to the E2 genotype, the effect of changing the concentration of growth regulators was more reduced than for E1. However, combining 2x NLN, 4% sucrose and 20% growth regulators was found strongly positive in comparison with the control condition (Figure 4C), as for E1. The number of calli larger than 1 mm showed no significant changes (Figure 4D). Therefore, increases in the concentration of NLN medium and sucrose together with reduced levels of growth regulators, were found positive to increase the efficiency of embryogenesis induction in the F1 hybrids.

The production of callus in DH36 control cultures was found much higher than in the hybrid genotypes (11.4 and 10.2 times superior to E1 and E2, respectively). DH36 reacted differently to medium modifications in qualitative terms too (Figures 4E and 4F). Increased concentrations of NLN medium and sucrose increased 2.1 times the total number of calli, but affected negatively the number of calli larger than 1 mm. A reduced concentration of growth regulators showed no positive effect on the total number of calli, but the number of calli larger than 1 mm was 1.4 times higher. The combination of 2x NLN, 4% sucrose and 20% growth regulators was negative for the number of call larger than 1 mm. These results indicated that the hybrid genotypes showed a similar response to medium modifications, in general terms, whereas the response of the DH line was remarkably different.

Our next goal was to determine to what extent the observed effects were due to the changes in sucrose, in NLN medium or in both. The highest concentration of sucrose (5%) improved the total number of calli in E1 and E2 (5.4 and 66.6 times more, respectively), but not in DH36 (Figure 5A). There were no changes in the number of calli larger than 1 mm for E1 and E2, but this parameter was reduced in DH36 (Figure 5B). We tested even higher concentrations of sucrose (10%), but we found that they inhibited induction in all cases (data not shown). Therefore, an increase of sucrose to 5% clearly benefited the embryogenic response of hybrids, but not of DH36. This indicated that the positive effects previously observed in DH36 would come from the increase in concentration of NLN medium. To confirm it, we evaluated in DH36 the effect of combining 2x NLN concentrations

with 100% and 20% levels of growth regulators. The first combination provided the highest yield in total callus (Figure 6A), whereas the second provided the highest yield in number of calli larger than 1 mm (Figure 6B). Again, the response of DH36 to modifications in the composition of the culture medium was remarkably different from that of E1 and E2 hybrids.

Response to different plating densities

Finally, we tested whether cell density influences microspore induction and growth, as well as callus production. For this, we worked only with DH36, discarding E1 and E2 due to their low response to induction. Microspore densities below the standard of 5×10^5 microspores/mL were positive in terms of number of total calli and the number of calli larger than 1 mm (Figure 7A). At 2×10^5 microspores/mL, the number of total calli counted was the highest (1.4 times more than the control), and increased the number of calli larger than 1 mm as well. However, the highest augment (1.6 times more than control) was observed for 3×10^5 microspores/mL (Figure 7B). A density of 1×10^5 microspores/mL notably diminished the total number of calli (7.7 times less than control). A density of 5×10^4 microspores/mL was insufficient to produce any calli in any culture plate (Figure 7A). On the contrary, the response of densities higher than the standard, like 1×10^6 microspores/mL was not significantly different from control. At 2×10^6 microspores/mL, there was not response since bacterial contamination was systematically observed in all culture plates (data not shown). Altogether, eggplant microspores showed a different response when cultured at different densities. Typically, the response was higher at densities below the 5×10^5 standard, but within a range, since below 5×10^4, the response is inhibited.

Discussion

As opposed to boron addition, light conditions of donor plants influence microspore viability

The relationship between microspore embryogenesis and growth conditions of donor plants has been commonly acknowledged. However, not much work has been focused on the determination of their best conditions. For example, despite that the levels of certain elements is known to affect the general status of the plant, the only element whose relationship with embryogenic competence of microspores has been studies is nitrogen (Tsay 1981; 1982). The exogenous application of boron to leaves is known to have a specific effect in pollen, increasing its quality and viability, both *in vivo* and *in vitro*, in other solanaceae such as pepper (Peñaloza and Toloza 2018). Since more viable

microspores/pollen imply more embryos potentially produced and therefore increased efficiencies, it seemed reasonable to test whether this could have a similar effect in a related species such as eggplant. Unfortunately, this was not the case, at least in our eggplant genotypes. No positive effect in microspore/pollen viability was observed, being even negative at the highest concentration. Thus, we must discard this treatment to improve the efficiency of the process.

Seasonal variations affect the androgenic response of plants. Indeed, photoperiod, light intensity and temperature are factors proposed to be involved (reviewed in Dunwell 2010). In the particular case of eggplant, the scarce data available are restricted to the summer-autumn transition, where it was reported that the highest response was found between September and October (Tuberosa et al. 1987). Based on this, it was proposed that, at least for the Mediterranean climate, the most favorable periods would be spring and autumn (Rotino 1996). Among the different parameters included in "seasonal variation", it is known that temperature to which donor plants are exposed may modulate the androgenic response (Dunwell 1976; Dunwell 2010). It is reasonable to think that when donor plants are continuously exposed to temperatures close to those used to induce embryogenesis, they trigger a previous stress response that desensitizes microspores against the subsequent embryogenesis-inducing heat shock treatment. Far from this stressing range, this relationship is less clear. We showed that at least for one genotype (E2), viability and number of calli correlated with temperature and illuminance. However, the lack of uniformity between E1 and E2 makes us think that at most, this could be a genotype-dependent relationship that cannot be extended beyond eggplant E2.

In our experiments we found that light may affect the response even more than temperature. Response is affected by reducing the initial viability of isolated microspores. Since less microspores are alive, less embryos can be induced. We also refined this notion, revealing that the light immediately received by plants during the few light hours from dawn to the moment of bud excision did not correlate with viability. Similarly, the light received during the week before excision did not correlate either. In contrast, the period spanning along two days before bud excision and microspore isolation appeared critical for microspore viability. Illuminance has been shown important in some species for proper flower development (Saxena and Johansen 1987) and even for pollen viability, being this trait genotype-dependent (Dutta et al. 2017). In tobacco, different light intensities were related to different effects in MDE induction and progression (Dunwell 1976). Since nothing is known in eggplant about it, we could speculate that flower development and in particular microspore development in eggplant would also be affected by changes in illuminance. This way, higher illuminances would somehow preclude a proper development of eggplant microspores, thus reducing their viability. This, however, must be studied

more in depth and experimentally confirmed. The precise mechanism by which changes in illuminance would affect microspore viability would also be interesting to elucidate.

In conclusion, our results indicate that variations in light are related to viability of *in vivo* microspores, which will obviously have a reflection in the amount of callus produced. These variations appear differently perceived by donor plants of different genotypes, making them sensitive to different extents. Therefore, it would be advisable to evaluate, when possible, such sensitivity before working with a new eggplant material.

DH36 does not respond to changes in culture medium conditions as hybrid genotypes do

The source of carbon for cell metabolism and its concentration in the culture medium is an important parameter to consider in any *in vitro* culture technique. Different studies have addressed this issue in different species (reviewed in Seguí-Simarro 2010). In highly responding genotypes, the medium used to induce embryogenesis typically includes high concentrations of sucrose. This is the case of *Brassica napus*, where culture medium includes 13% sucrose (Custers 2003), tobacco with 8.6% sucrose (Touraev et al. 1996a), maize with 5.1% sucrose (Gaillard et al. 1991) or hot pepper, with 10% sucrose (Kim et al. 2008). In the *in vitro* culture of eggplant anthers, 12% sucrose is typically used at the initial culture stage (Chambonnet 1988). Instead, the liquid medium used for microspore culture contains 2% sucrose (Corral-Martínez and Seguí-Simarro 2012; 2014; Miyoshi 1996). This led us to the hypothesis that perhaps, higher levels of sucrose could positively influence callus production. Our results were not homogeneous though. When we used high sucrose levels together with 2x NLN and 20% growth regulators, more calli were obtained in E1 and E2, but not in DH36. In DH36, the best results came from the use of high sucrose together with 2x NLN. Experiments increasing sucrose only showed a strong positive effect in E1 and E2, but not in DH36. Therefore, it appears that that increasing the concentration of NLN medium is positive for DH36, but not for hybrid genotypes. On the contrary, an increase in the concentration of sucrose greatly influences the response of hybrid genotypes, but not of DH36.

As to growth regulators, the pioneering work of Miyoshi (1996) showed the best results with 0.5 mg/l NAA and 0.5 mg/l BAP. More recent works suggested that eggplant microspores are more sensitive to absolute concentrations of growth regulators than to the relative ratios between them. Indeed, Corral-Martínez and Seguí-Simarro (2014) reported more calli with the use of reduced NAA and BAP concentrations. Consistent with this, the present study found that reduced levels clearly have a strong and positive influence in hybrid genotypes, but not in DH36. This suggests that the response of DH36 to changes in the composition of the medium is opposite to the response

of hybrid genotypes. Apparently, this line reached a peak of performance, difficult to overcome by modifications of the medium.

Lower plating density reduces competence and allows for a higher efficiency

Setting up the most suitable plating density is a key parameter for most if not all *in vitro* cell cultures. In microspore cultures, this parameter may have an effect in the number of calli or embryos produced, as well as in the quality and velocity of development (Camacho-Fernández et al. 2018; Seguí-Simarro et al. 2011). Indeed, each species has different requirements in terms of plating densities. Examples of optimal densities are 1.4×10^6 in apple (Höfer 2004) , 1×10^6 in barley (Esteves et al. 2014), 1×10^5 in wheat or pepper (Kim et al. 2013; Sinha and Eudes 2015), 5×10^4 in tobacco (Touraev and Heberle-Bors 2003), or 4×10^4 microspores/mL in maize and rapeseed (Aulinger 2002; Robert et al. 2015). To ascertain whether microspore plating density influences the androgenic response, we evaluated a broad range of densities, finding that lower densities (2×10^5-3×10^5 microspores/mL) were the best for higher rates of callus production and callus growth. On the other hand, higher densities had a negative effect. This might be explained by two hypothesis: (1) an excessive accumulation of potentially toxic and/or inhibitory substances excreted by cells to the culture medium, or (2) the competition of cells for resources and space. Our experiments suggested the second option, because in most of them, an inverse correspondence between callus number and callus growth was found in the three genotypes used, but especially in DH36, where more calli are formed. Interestingly, the assay with the highest number of calli in DH36 was the use of 2x NLN medium and 2x sucrose (Figure 4E). Duplicating the availability of nutrients duplicated the callus yield. Besides, calli are induced structures, already formed, not supposedly affected by the excretion of putative inductive/inhibitory substances. In other words, when embryogenesis starts, microspore-derived structures are forced to share and therefore to compete for limited resources and space, reducing the growth rate.

We also demonstrated that there is a minimal threshold of microspore density below which embryogenesis is not induced. Similar findings were described by Kim et al. (2008) in microspore cultures of hot pepper. It is known that embryogenic microspores excrete different factors that may have a positive influence in neighbor microspores, promoting their entry into embryogenesis and/or growth (Borderies et al. 2004; Paire et al. 2003; Żur et al. 2015). At very low microspore densities, these factors may become too diluted to have an effect. It was also interesting to note the systematic appearance of bacterial contamination at very high densities (10x the optimal density proposed hereby). Most likely, endogenous bacteria typically present in the intercellular spaces of different

plant tissues and organs, including anther walls, are responsible for this. These endophytes cannot be eliminated with the routine surface disinfection of buds or anthers, and may proliferate if the initial inoculum is high enough. Higher microspore densities would carry higher levels of bacterial inoculum, A similar phenomenon has been reported in pepper, which makes mandatory the routine use of antibiotics (Lantos et al. 2012; Parra-Vega and Seguí-Simarro 2013; Supena et al. 2006).

Concluding remarks

We used for this work two hybrids and a DH line. In our experiments, callus production was always higher (from 10 to 85x higher) in DH36 than in hybrids. The DH36 line showed also four times more response than the hybrid used to develop this line (Rivas-Sendra et al. 2017b). Ideally, DH36 may produce up to ~450 calli/mL, which implies a minimum of ~450 initially induced embryos/mL. This yield is at the levels found in the best responding lines of model species. We also showed that the response of DH36 was quite difficult to enhance by adding modifications to the medium, since many of these modifications negatively influenced callus growth, as opposed to their effect in hybrid genotypes. Thus, the nature of DH36, whose homozygous alleles were selected according to their high response to induction of embryogenesis, could be approaching the performance of this line to a maximum level, making it hardly sensitive to minor modifications of the medium. In turn, we were able to increase the efficiency of 'Bandera', the hybrid line from which DH36 was derived, by changing medium composition (Corral-Martínez and Seguí-Simarro 2012; 2014).

In most of the highly responding model systems, exogenous growth regulators are not needed to induce embryogenesis. Examples of this are rapeseed (Custers 2003), maize (Gaillard et al. 1991), tobacco (Touraev et al. 1996a) and wheat (Touraev et al. 1996b). Embryos are induced in these species using no growth regulators, or using them at very low concentrations. This points to an endogenous production of growth regulators enough for their requirements. In recalcitrant species, the scenario is the opposite, it is essential to add exogenous growth regulators to promote cell division and/or morphogenesis (Seguí-Simarro et al. 2011). The null or negative response of DH36 to changes in the levels of growth regulators would suggest that this line has an endogenous balance of growth regulators better fitted to embryogenesis induction under the standard concentrations of growth regulators in the culture medium used. This, in turn, would be an indirect support to the notion that DH36 is sensitive but less dependent than hybrid genotypes on the addition of exogenous growth regulators. This is consistent with the fact that DH36 was selected for high response in a specific culture medium containing a fixed composition in term of growth regulators

(0.5 mg/l NAA and 0.5 mg/l BAP). The possibility of selecting lines with different androgenic response is an additional evidence of the genetic control of this trait.

However, the levels of growth regulators may not necessarily be the same at all culture stages. Indeed, unbalanced growth regulator levels may affect negatively to the progression of embryos, thereby causing the transformation of globular embryos into calli, the main bottleneck in eggplant microspore culture (Seguí-Simarro 2016). This would surely be the next step to approach DH36 to model lines of other species.

Acknowledgements

This work was supported by grant AGL2017-88135-R to JMSS from Spanish MICINN, respectively, jointly funded by FEDER. ARS and CCF were supported by predoctoral fellowships from the FPI Programs of Universitat Politècnica de València and Generalitat Valenciana, respectively.

Author contribution

JMSS designed the work. PCM, ARS, RP and CCM performed the experiments. JMSS and ARS wrote the manuscript.

Conflict of interest

The authors declare no conflict of interest

References

Abdollahi MR, Corral-Martinez P, Mousavi A, Salmanian AH, Moieni A, Seguí-Simarro JM (2009) An efficient method for transformation of pre-androgenic, isolated *Brassica napus* microspores involving microprojectile bombardment and *Agrobacterium*-mediated transformation. Acta Physiol Plant 31: 1313-1317

Aulinger IE (2002) Combination of in vitro androgenesis and biolistic transformation: an approach for breeding transgenic maize (*Zea mays* L.) lines. Swiss Federal Institute of Technology, Zurich, p 115

Borderies G, le Bechec M, Rossignol M, Lafitte C, Le Deunff E, Beckert M, Dumas C, Matthys-Rochon E (2004) Characterization of proteins secreted during maize microspore culture: arabinogalactan proteins (AGPs) stimulate embryo development. Eur J Cell Biol 83: 205-212

504 Bueno MA, Gómez A, Sepúlveda F, Seguí-Simarro JM, Testillano PS, Manzanera JA, Risueño MC
505 (2003) Microspore-derived embryos from *Quercus suber* anthers mimic zygotic embryos
506 and maintain haploidy in long-term anther culture. J Plant Physiol 160: 953-960
507 Camacho-Fernández C, Hervás D, Rivas-Sendra A, Marín MP, Seguí-Simarro JM (2018)
508 Comparison of six different methods to calculate cell densities. Plant Methods 14: 30
509 Corral-Martínez P, Seguí-Simarro JM (2012) Efficient production of callus-derived doubled
510 haploids through isolated microspore culture in eggplant (*Solanum melongena* L.).
511 Euphytica 187: 47-61
512 Corral-Martínez P, Seguí-Simarro JM (2014) Refining the method for eggplant microspore culture:
513 effect of abscisic acid, epibrassinolide, polyethylene glycol, naphthaleneacetic acid, 6-
514 benzylaminopurine and arabinogalactan proteins. Euphytica 195: 369-382
515 Custers J (2003) Microspore culture in rapeseed (*Brassica napus* L.). In: Maluszynski M, Kasha KJ,
516 Forster BP, Szarejko I (eds) Doubled haploid production in crop plants. Kluwer Academic
517 Publishers, Dordrecht, The Netherlands, pp 185-193
518 Chambonnet D (1988) Production of haploid eggplant plants. Bulletin interne de la Station
519 d'Amélioration des Plantes Maraichères d'Avignon-Montfavet, France: 1-10
520 Dunwell JM (1976) A comparative study of environmental and developmental factors which
521 influence embryo induction and growth in cultured anthers of *Nicotiana tabacum*. Environ
522 Exp Bot 16: 109-118
523 Dunwell JM (2010) Haploids in flowering plants: origins and exploitation. Plant Biotechnol J 8:
524 377-424
525 Dutta SS, Pale G, Pattanayak A, Aochen C, Pandey A, Rai M (2017) Effect of low light intensity on
526 key traits and genotypes of hilly rice (*Oryza sativa*) germplasm J Exp Biol Agric Sci 5: 463-
527 471
528 Esteves P, Clermont I, Marchand S, Belzile F (2014) Improving the efficiency of isolated
529 microspore culture in six-row spring barley: II-exploring novel growth regulators to
530 maximize embryogenesis and reduce albinism. Plant Cell Rep: In press
531 Gaillard A, Vergne P, Beckerte M (1991) Optimization of maize microspore isolation and culture
532 conditions for reliable plant regeneration. Plant Cell Rep 10: 55-58
533 Höfer M (2004) In vitro androgenesis in apple—improvement of the induction phase. Plant Cell
534 Rep 22: 365-370
535 Jouannic S, Champion A, Seguí-Simarro JM, Salimova E, Picaud A, Tregear J, Testillano P,
536 Risueno MC, Simanis V, Kreis M, Henry Y (2001) The protein kinases AtMAP3Kepsilon1

and BnMAP3Kepsilon1 are functional homologues of S. pombe cdc7p and may be involved in cell division. Plant J 26: 637-649

Kim M, Jang I-C, Kim J-A, Park E-J, Yoon M, Lee Y (2008) Embryogenesis and plant regeneration of hot pepper (*Capsicum annuum* L.) through isolated microspore culture. Plant Cell Rep 27: 425-434

Kim M, Park E-J, An D, Lee Y (2013) High-quality embryo production and plant regeneration using a two-step culture system in isolated microspore cultures of hot pepper (*Capsicum annuum* L.). Plant Cell Tissue Organ Cult 112: 191-201

Lantos C, Juhasz AG, Vagi P, Mihaly R, Kristof Z, Pauk J (2012) Androgenesis induction in microspore culture of sweet pepper (*Capsicum annuum* L.). Plant Biotechnol Rep 6: 123-132

Liu L, Huang L, Li Y (2013) Influence of boric acid and sucrose on the germination and growth of areca pollen. American Journal of Plant Sciences 2013

Miyoshi K (1996) Callus induction and plantlet formation through culture of isolated microspores of eggplant (*Solanum melongena* L). Plant Cell Rep 15: 391-395

Paire A, Devaux P, Lafitte C, Dumas C, Matthys-Rochon E (2003) Proteins produced by barley microspores and their derived androgenic structures promote in vitro zygotic maize embryo formation. Plant Cell Tissue Organ Cult 73: 167-176

Parra-Vega V, Seguí-Simarro JM (2013) Improvement of an isolated microspore culture protocol for Spanish sweet pepper (*Capsicum annuum* L.). In: Lanteri S, Rotino GL (eds) Breakthroughs in the genetics and breeding of *Capsicum* and *Eggplant*. Universita degli Studi di Torino, Torino, Italy, pp 161-168

Peñaloza P, Toloza P (2018) Boron increases pollen quality, pollination, and fertility of different genetic lines of pepper. Journal of Plant Nutrition 41: 969-979

Rivas-Sendra A, Calabuig-Serna A, Seguí-Simarro JM (2017a) Dynamics of Calcium during In vitro Microspore Embryogenesis and In vivo Microspore Development in *Brassica napus* and *Solanum melongena*. Front Plant Sci 8: 1177

Rivas-Sendra A, Campos-Vega M, Calabuig-Serna A, Seguí-Simarro JM (2017b) Development and characterization of an eggplant (*Solanum melongena*) doubled haploid population and a doubled haploid line with high androgenic response. Euphytica 213: 89

Rivas-Sendra A, Corral-Martínez P, Camacho-Fernández C, Seguí-Simarro JM (2015) Improved regeneration of eggplant doubled haploids from microspore-derived calli through organogenesis. Plant Cell Tissue Organ Cult 122: 759-765

570 Rivas-Sendra A, Corral-Martínez P, Porcel R, Camacho-Fernández C, Calabuig-Serna A, Seguí-
571 Simarro JM (2019) Embryogenic competence of microspores is associated with their ability
572 to form a callosic, osmoprotective subintinal layer. J Exp Bot 70: 1267–1281
573 Robert HS, Grunewald W, Sauer M, Cannoot B, Soriano M, Swarup R, Weijers D, Bennett M,
574 Boutilier K, Friml J (2015) Plant embryogenesis requires AUX/LAX-mediated auxin influx.
575 Development 142: 702-711
576 Rotino GL (1996) Haploidy in eggplant. In: Jain SM, Sopory SK, Veilleux RE (eds) In vitro
577 haploid production in higher plants. Kluwer Academic Publishers, Dordrecht, The
578 Netherlands, pp 115-141
579 Salas P, Prohens J, Seguí-Simarro JM (2011) Evaluation of androgenic competence through anther
580 culture in common eggplant and related species. Euphytica 182: 261–274
581 Salas P, Rivas-Sendra A, Prohens J, Seguí-Simarro JM (2012) Influence of the stage for anther
582 excision and heterostyly in embryogenesis induction from eggplant anther cultures.
583 Euphytica 184: 235-250
584 Satpute G, Long H, Seguí-Simarro JM, Risueño MC, Testillano PS (2005) Cell architecture during
585 gametophytic and embryogenic microspore development in *Brassica napus*. Acta Physiol
586 Plant 27: 665-674
587 Saxena N, Johansen C (1987) Adaptation of chickpea and pigeonpea to abiotic stresses.
588 Proceedings of the Consultants' Workshop held at ICRISAT Center, India, 19-21 December
589 1984, ICRISAT, Patancheru, India
590 Seguí-Simarro JM (2010) Androgenesis revisited. Bot Rev 76: 377-404
591 Seguí-Simarro JM (2016) Androgenesis in solanaceae. In: Germanà MA, Lambardi M (eds) In vitro
592 embryogenesis. Springer Science + Business Media, New York, pp 209-244
593 Seguí-Simarro JM, Corral-Martínez P, Parra-Vega V, González-García B (2011) Androgenesis in
594 recalcitrant solanaceous crops. Plant Cell Rep 30: 765-778
595 Seguí-Simarro JM, Nuez F (2008) How microspores transform into haploid embryos: changes
596 associated with embryogenesis induction and microspore-derived embryogenesis. Physiol
597 Plant 134: 1-12
598 Sinha R, Eudes F (2015) Dimethyl tyrosine conjugated peptide prevents oxidative damage and
599 death of triticale and wheat microspores. Plant Cell Tissue Organ Cult: 1-11
600 Supena EDJ, Suharsono S, Jacobsen E, Custers JBM (2006) Successful development of a shed-
601 microspore culture protocol for doubled haploid production in Indonesian hot pepper
602 (*Capsicum annuum* L.). Plant Cell Rep 25: 1-10

603 Touraev A, Heberle-Bors E (2003) Anther and microspore culture in tobacco. In: Maluszynski M,
604 Kasha KJ, Forster BP, Szarejko I (eds) Doubled haploid production in crop plants. Kluwer
605 Academic Publishers, Dordrecht, The Netherlands, pp 223-228
606 Touraev A, Ilham A, Vicente O, Heberle-Bors E (1996a) Stress-induced microspore embryogenesis
607 in tobacco: an optimized system for molecular studies. Plant Cell Rep 15: 561-565
608 Touraev A, Indrianto A, Wratschko I, Vicente O, Heberle-Bors E (1996b) Efficient microspore
609 embryogenesis in wheat (*Triticum aestivum* L.) induced by starvation at high temperatures.
610 Sex Plant Reprod 9: 209-215
611 Tsay H-S (1981) Effects of nitrogen supply to donor plants on pollen embryogenesis in cultured
612 tobacco anthers. J Agric Res China 30: 5-13
613 Tsay H-S (1982) Microspore development and haploid embryogenesis of anther culture with five
614 nitrogen doses to the donor tobacco plants. J Agric Res China 31: 1-13
615 Tuberosa R, Sanguineti MC, Toni B, Cioni F (1987) Ottenimento di aploidi in melanzana (*Solanum*
616 *melongena* L.) mediante coltura di antere. Sementi Elette 3: 9-14
617 Żur I, Dubas E, Krzewska M, Janowiak F (2015) Current insights into hormonal regulation of
618 microspore embryogenesis. Front Plant Sci 6

619

620

Tables

Table 1: R^2 coefficients for the correlations between temperature, light viability, microspore divisions and calli produced in the E1 genotype. Coefficients in bold numbers represent high correlations between both variables. da: days after microspore isolation. db: days before microspore isolation. See text for further details.

	Temperature				Light			
	0 db	1 db	2 db	7 db	0 db	1 db	2 db	7 db
Viability (0 da)	0.36	0.54	0.21	0.44	0.35	**0.97**	**0.95**	0.16
Viability (3 da)	0.09	0.01	0.20	0.69	0.08	0.07	0.03	0.52
Divisions (7 da)	0.04	0.15	0.05	0.02	0.02	0.22	0.25	0.19
Calli (30 da)	0.00	0.02	0.18	0.37	0.67	0.43	0.37	0.41

Table 2: R^2 coefficients for the correlations between temperature, light, viability, microspore divisions and calli produced in the E2 genotype. Coefficients in bold numbers represent high correlations between both variables. da: days after microspore isolation. db: days before microspore isolation. See text for further details.

	Temperature				Light			
	0 db	1 db	2 db	7 db	0 db	1 db	2 db	7 db
Viability (0 da)	0.18	**0.99**	0.00	0.16	0.06	**0.98**	**0.98**	0.00
Viability (3 da)	0.19	**0.95**	0.21	0.39	0.02	**0.98**	**0.98**	0.07
Divisions (7 da)	0.00	0.55	0.19	0.06	0.00	0.46	0.46	0.35
Calli (30 da)	0.17	0.11	0.29	**0.98**	0.37	0.17	0.17	**0.93**

637 **Figure legends**

638

639 **Figure 1.** Effect of greenhouse temperature and light in the viability of *in vivo* microspores. **A**
640 illustrates the fluctuations of temperature and light in the greenhouse where E1 and E2 donor plants
641 were grown during the months from January to September. **B, C:** Microspore viabilities for the
642 different microspore cultures just upon isolation from anthers (day 0) and after the heat shock
643 inductive treatment (day 3), and microspore divisions after 7 days of culture, for E1 **(B)** and E2 **(C)**
644 genotypes. Viabilities and divisions are expressed as percentages from the total of microspore
645 counted ± standard deviation.

646

647 **Figure 2.** Application of boron to donor plants. A and B show images of FDA staining of
648 microspores from control plants (A) and from plants treated with 200 mg/L of boron (B). C:
649 Percentages of viable microspores for the control and the three boron concentrations applied. Bars
650 represent standard deviation. Different letters represent statistically significant differences
651 according to the LSD test with $p \leq 0.05$. D: Donor plants after three weeks of treatment with boron:
652 Bars: 50 µm.

653

654 **Figure 3.** Eggplant microspore cultures. A: Freshly isolated microspores, just after inoculation for
655 *in vitro* culture. B: Seven-day-old cultures. Note the growth of induced, proliferating microspores
656 (arrowheads). C: Thirty-day-old cultures, where microspore-derived calli are clearly visible. D:
657 Calli cultured in solid medium. In some of them, shoots are induced to regenerate. Note that other
658 calli do not regenerate shoots, and other die. E: Isolated, rooting shoot. F: Fully rooted, entire DH
659 plant. Bars: A-C: 50 µm. D, E: 1 cm.

660

661 **Figure 4.** Effect of increased concentrations of NLN medium and reduced levels of growth
662 regulators in E1, E2 and DH36. **A, C** and **E:** Total calli produced at day 30. **B, D** and **F:** Calli larger
663 than 1 mm produced at day 30. Note the different scale in **E** and **F**. In each chart, the first column
664 corresponds to control conditions. Bars represent standard deviation. Different letters indicate
665 statistically significant differences according to the LSD test with $p \leq 0.05$. **nt:** not tested.

666

667 **Figure 5.** Effect of increased concentration of sucrose in E1, E2 and DH36. **A:** Total calli produced
668 at day 30. **B:** Calli larger than 1 mm produced at day 30. For each genotype, the first column
669 corresponds to control conditions. Bars represent standard deviation. Different letters indicate
670 statistically significant differences according to the LSD test with $p \leq 0.05$.

671

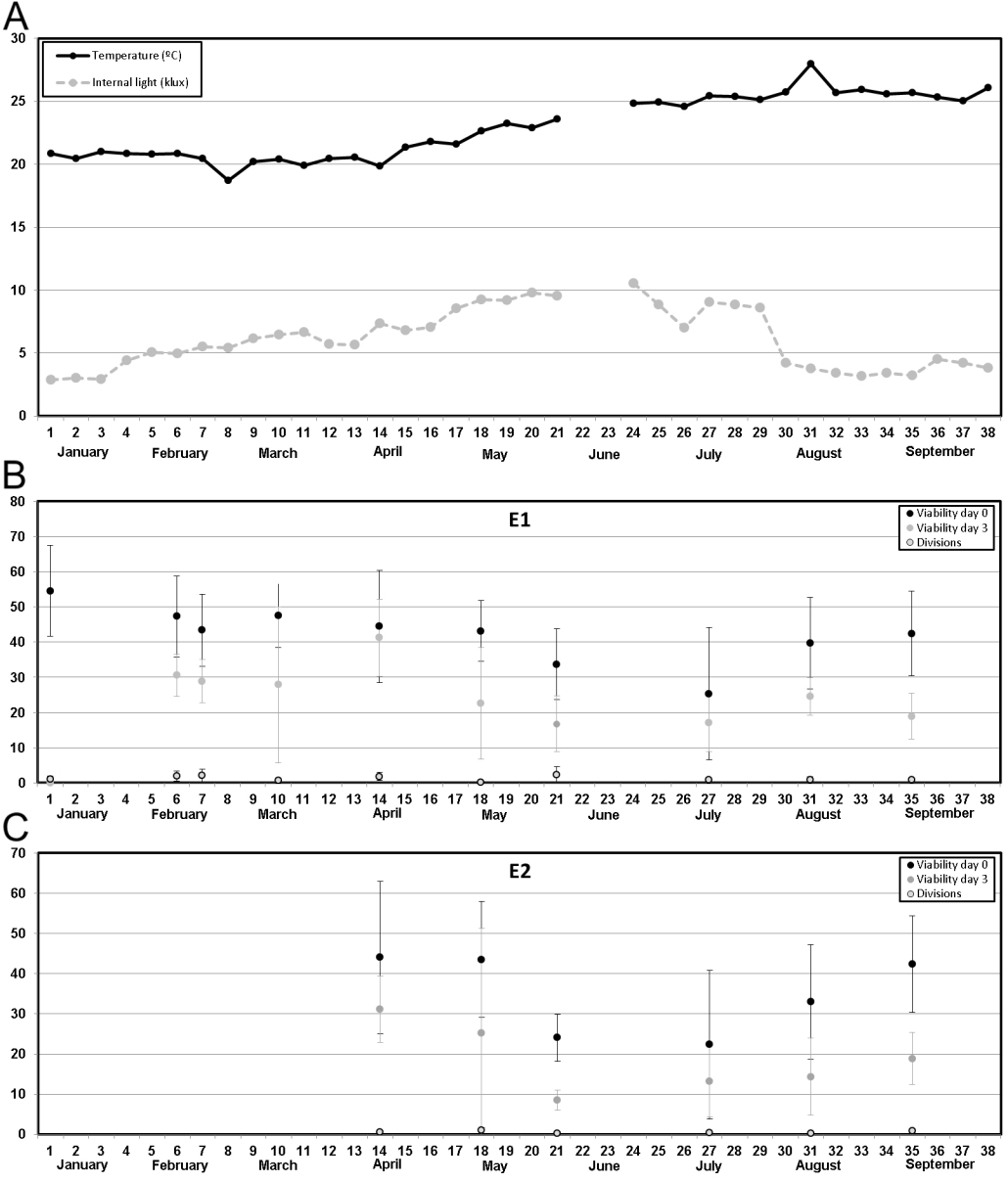
672 **Figure 6.** Effect of increased concentration of NLN medium in DH36. Total calli produced at day
673 30. **B:** Calli larger than 1 mm produced at day 30. For each genotype, the first column corresponds
674 to control conditions. Bars represent standard deviation. Different letters indicate statistically
675 significant differences according to the LSD test with $p \leq 0.05$.

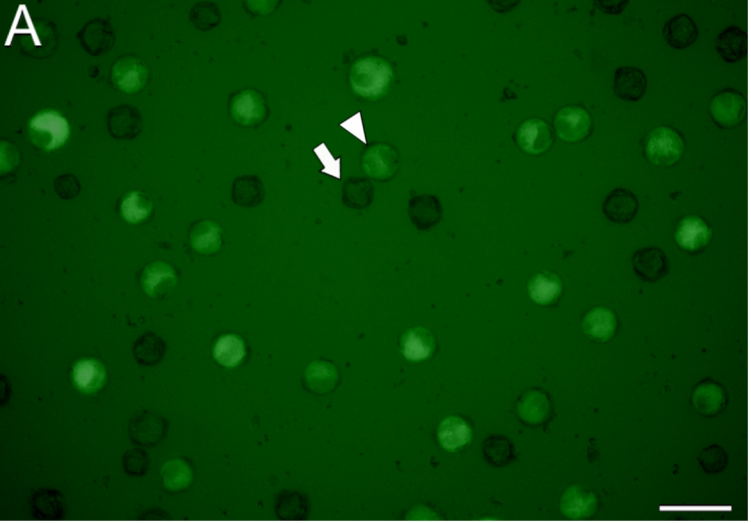
676

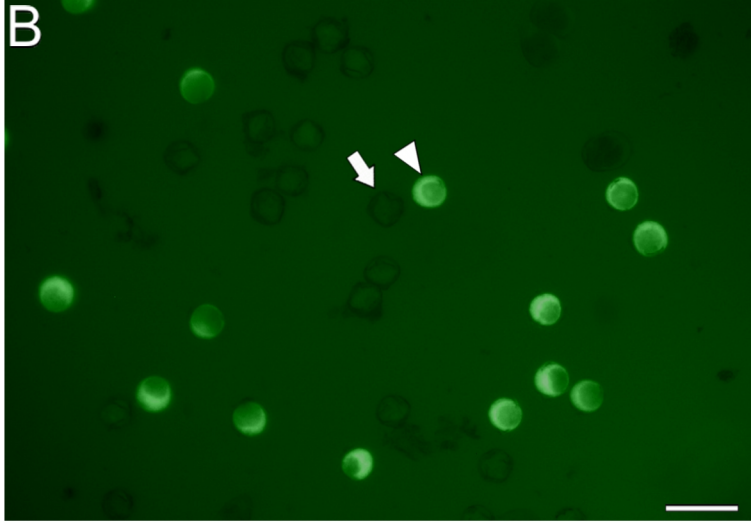
677 **Figure 7.** Effect of different plating densities in microspore cultures of the DH36 line. Total calli
678 produced at day 30. **B:** Calli larger than 1 mm produced at day 30. To be able to compare among
679 densities, efficiency is expressed as calli per 5×10^5 plated microspores (calli/5×10^5 msp). Bars
680 represent standard deviation. Different letters indicate statistically significant differences according
681 to the LSD test with $p \leq 0.05$.

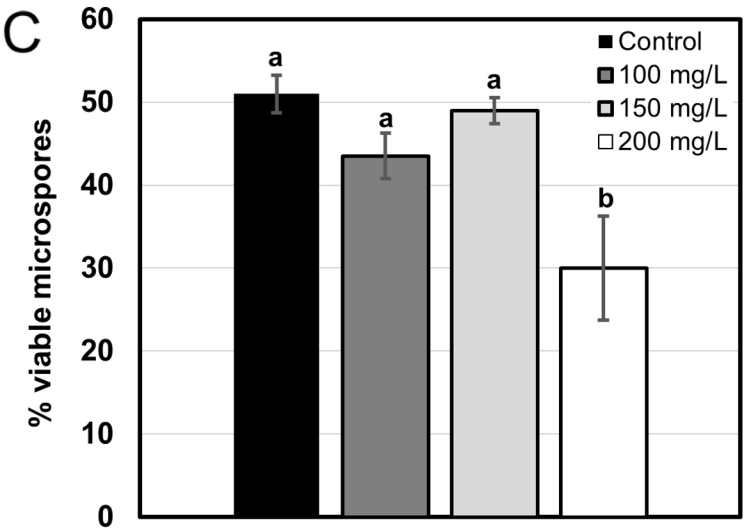
682

683

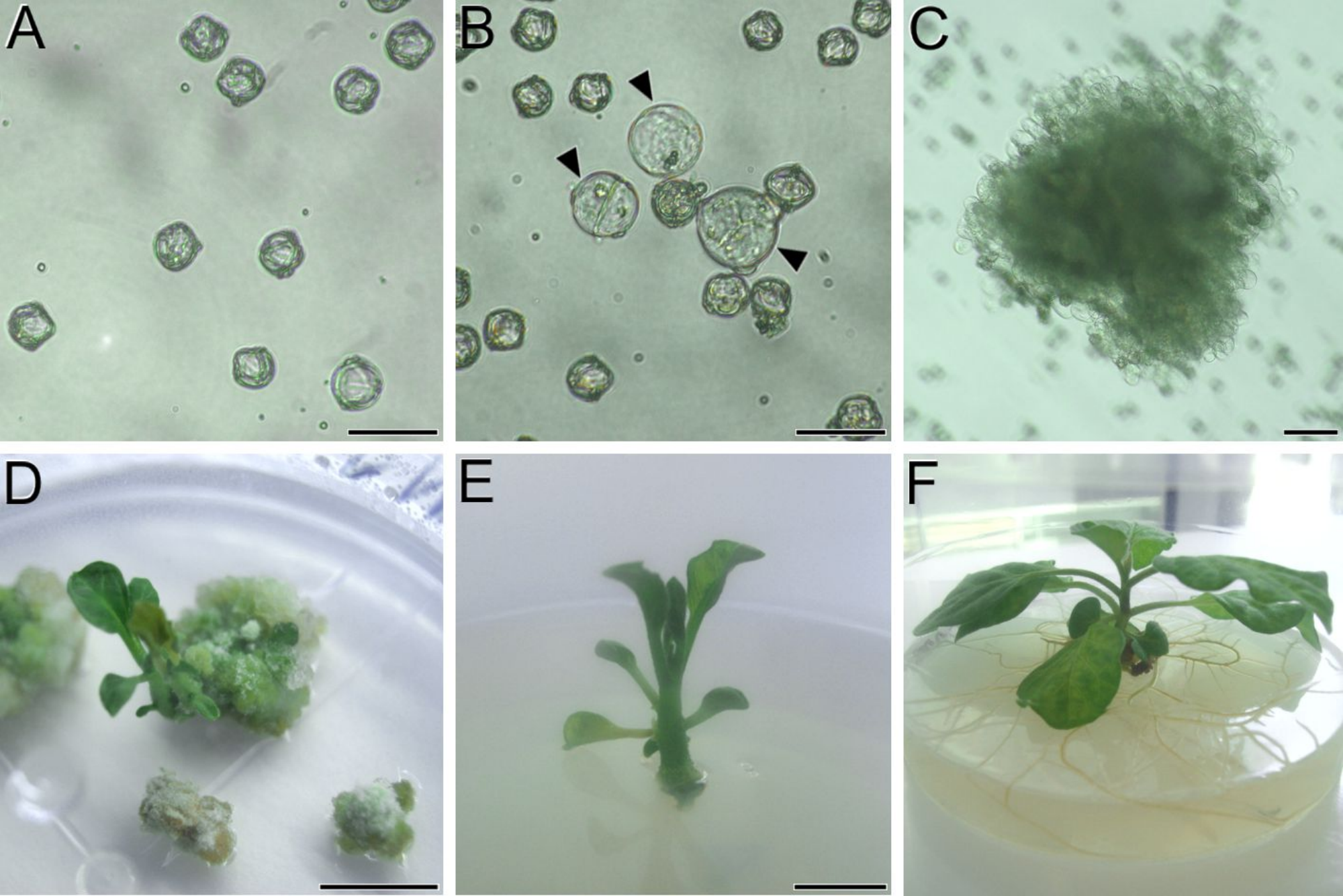

A
Temperature (ºC)
Internal light (klux)
30
25
20
15
10
5
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38
January
February
March
April
May
June
July
August
September
B
E1
Viability day 0
Viability day 3
Divisions
80
70
60
50
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38
January
February
March
April
May
June
July
August
September
C
E2
Viability day 0
Viability day 3
Divisions
70
60
50
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38
January
February
March
April
May
June
July
August
September

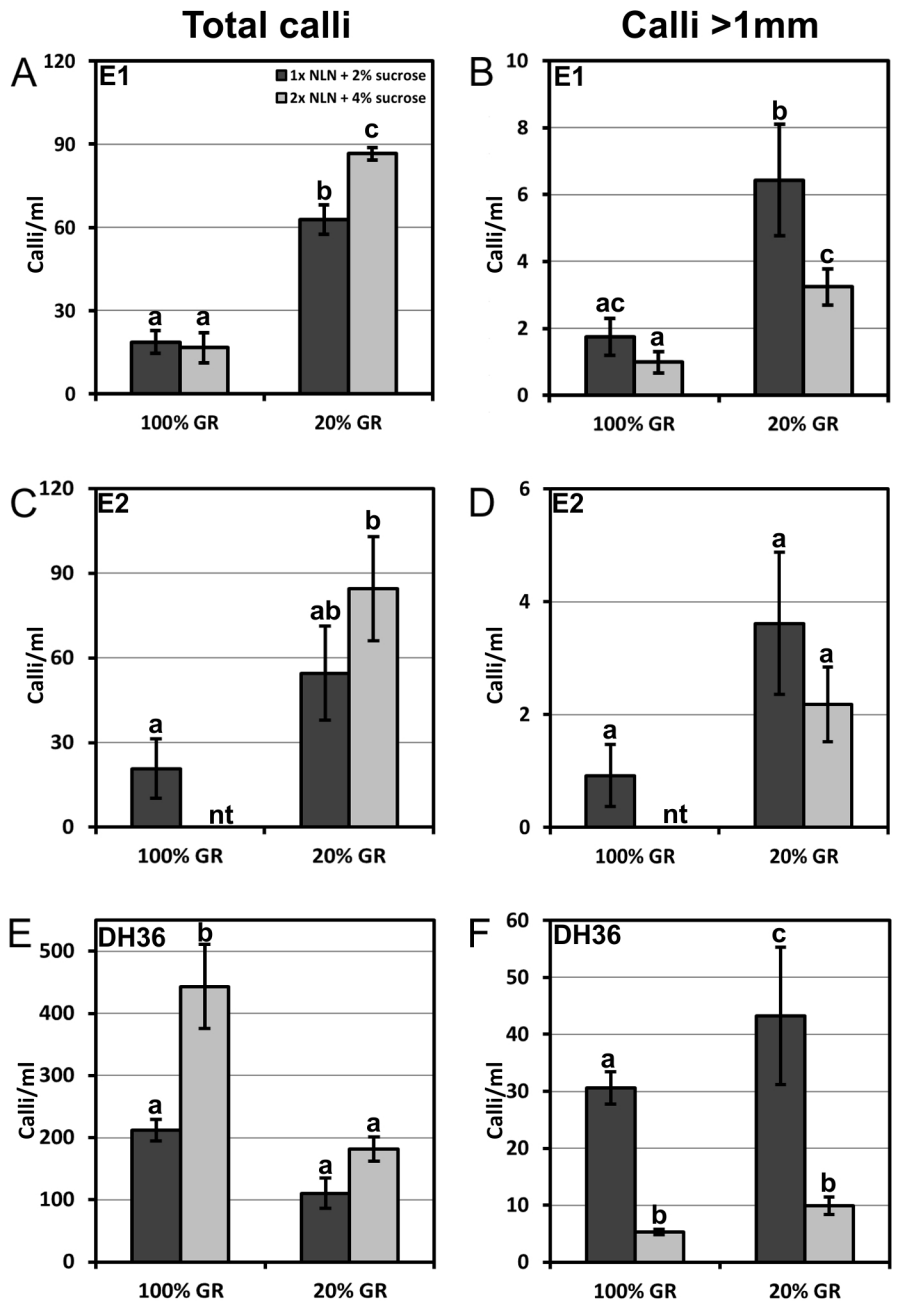

A

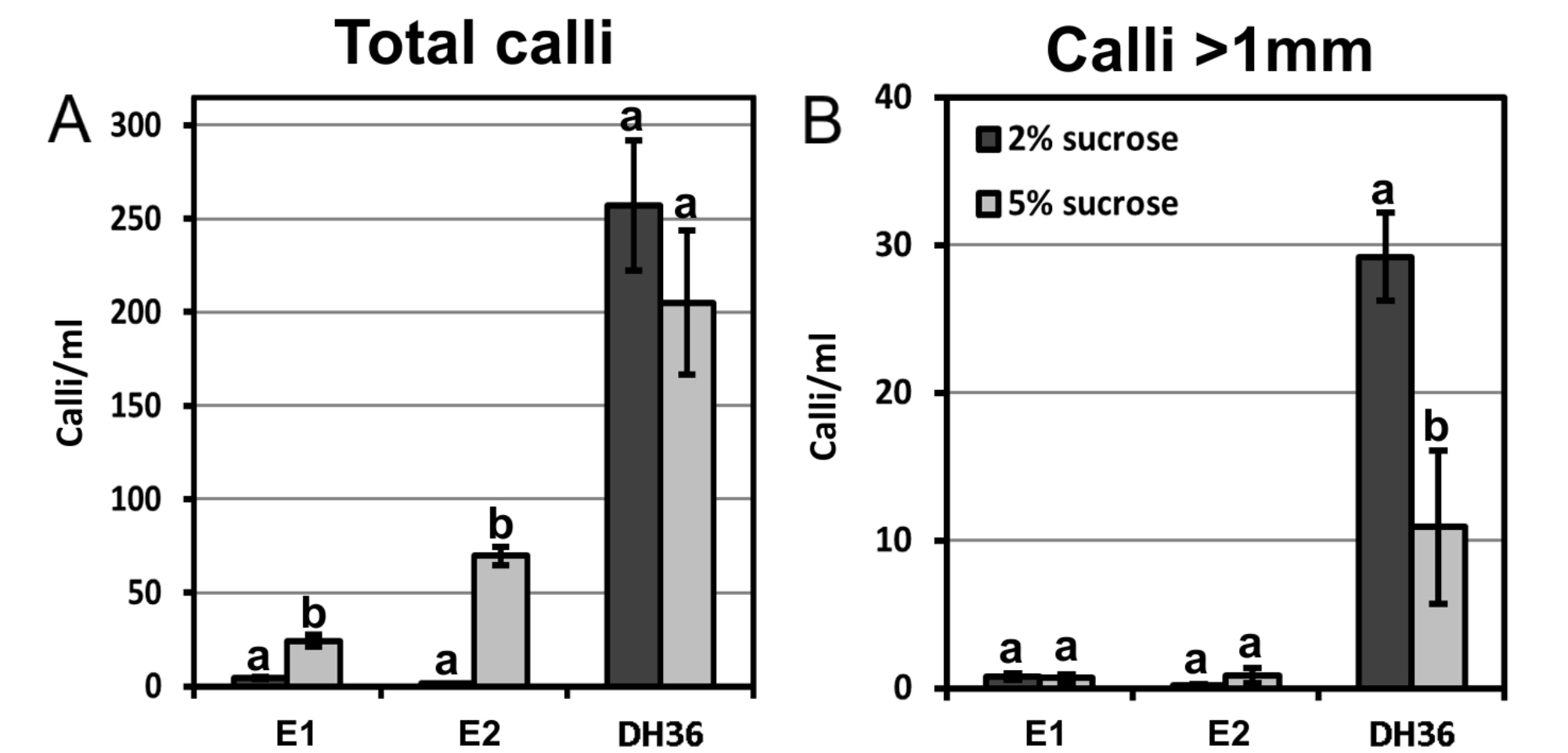

B

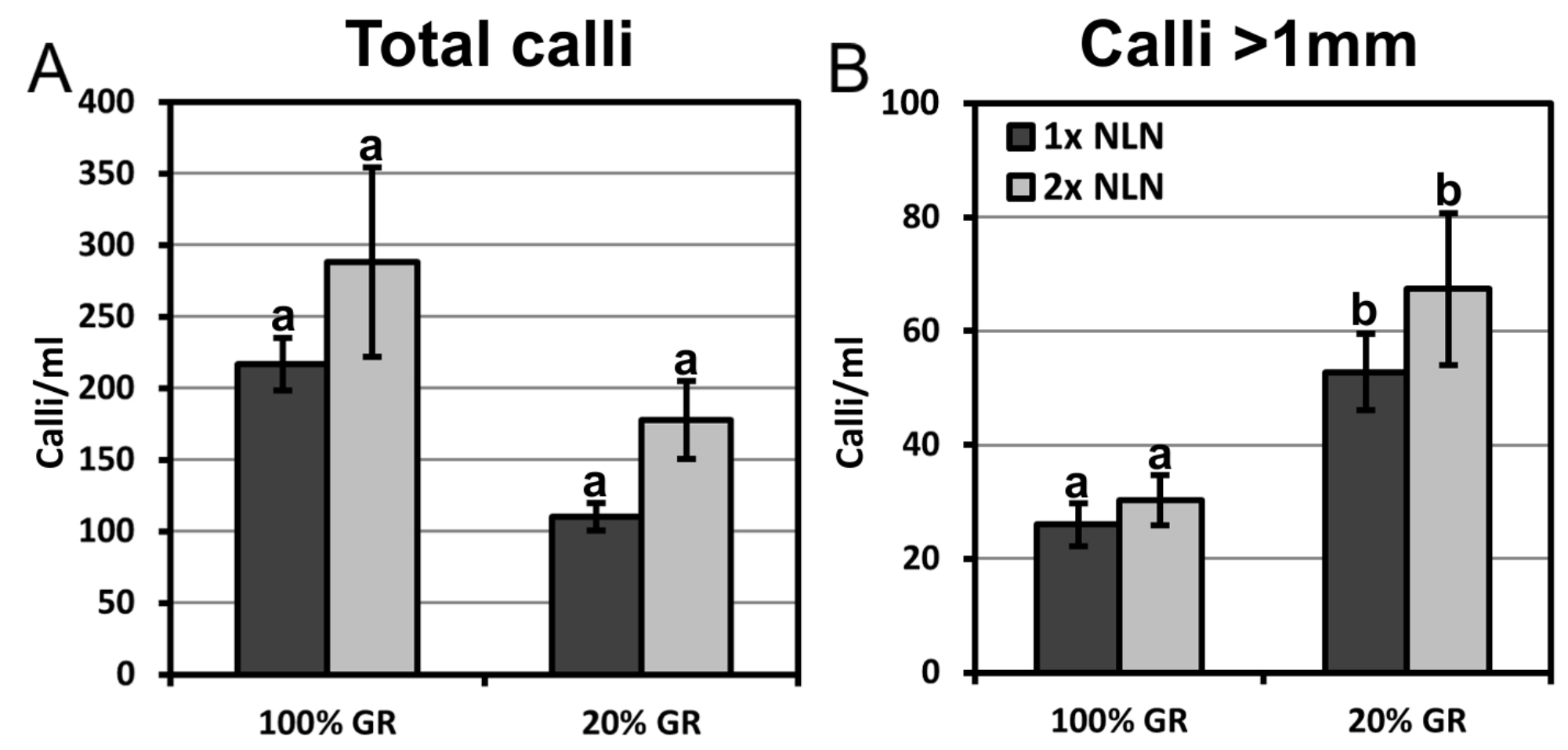

C
% viable microspores
60
50
40
30
20
10
0
a
a
a
b
Control
100 mg/L
150 mg/L
200 mg/L

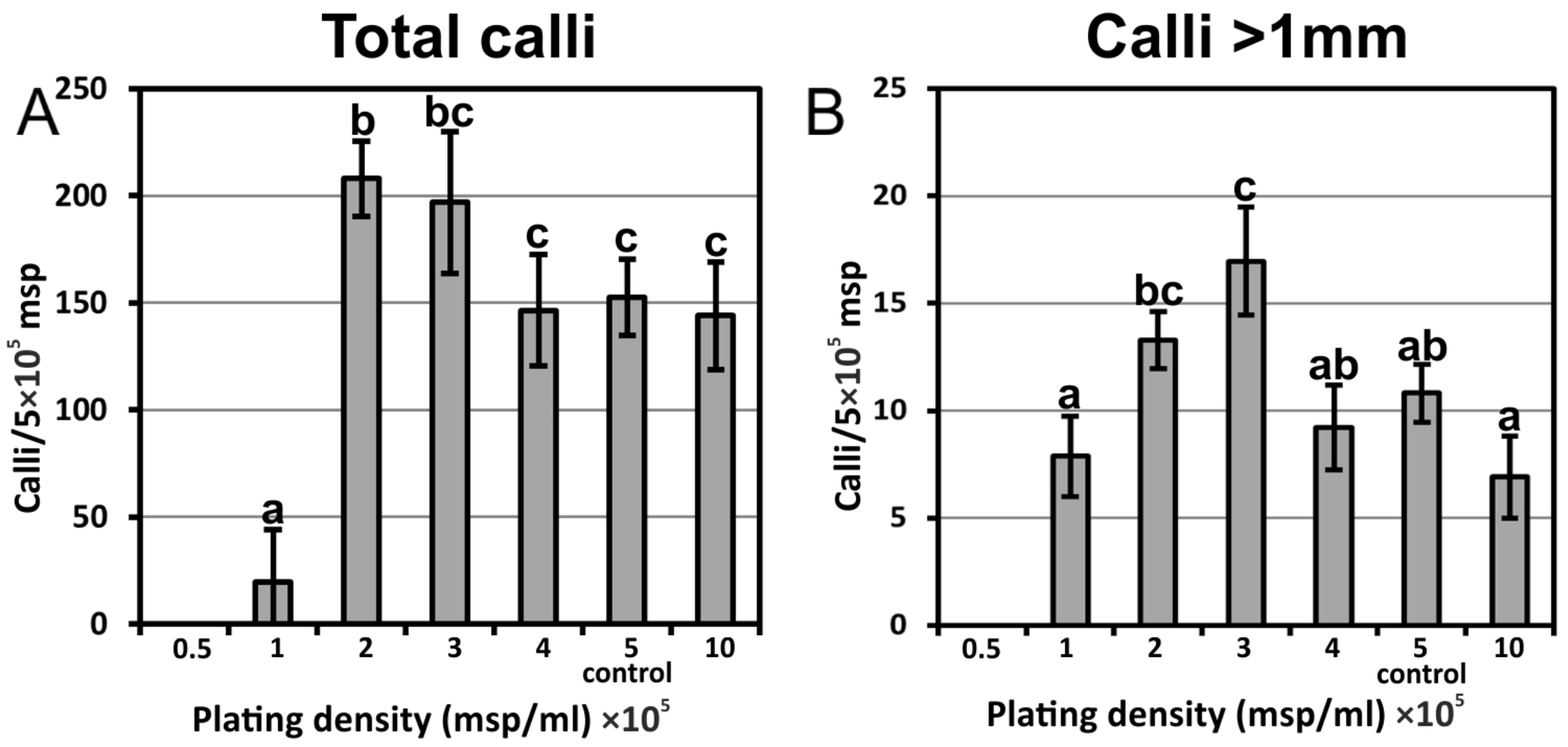
D


A
B
C
D
E
F


Total calli
Calli >1mm
A
E1
1x NLN + 2% sucrose
2x NLN + 4% sucrose
Calli/ml
120
90
60
30
0
a
a
b
c
100% GR
20% GR
B
E1
10
8
6
4
2
0
ac
a
b
c
C
E2
ab
nt
D
E2
E
DH36
500
400
300
200
100
F
DH36
60
50
40
30
20
10


Total calli
A
Calli/ml
300
250
200
150
100
50
0
a
b
a
b
a
a
E1
E2
DH36
Calli >1mm
B
Calli/ml
40
30
20
10
0
2% sucrose
5% sucrose
a
a
a
a
a
b
E1
E2
DH36


A
Total calli
Calli/ml
400
350
300
250
200
150
100
50
0
a
a
a
a
100% GR
20% GR
B
Calli >1mm
Calli/ml
100
80
60
40
20
0
1x NLN
2x NLN
a
a
b
b
100% GR
20% GR


Total calli
A
250
200
150
100
50
0
Calli/5×10^5 msp
a
b
bc
c
c
c
0.5
1
2
3
4
5
control
10
Plating density (msp/ml) ×10^5
Calli >1mm
B
25
20
15
10
5
0
Calli/5×10^5 msp
a
bc
c
ab
ab
a
0.5
1
2
3
4
5
control
10
Plating density (msp/ml) ×10^5