Studies on Sporophytic Development of Cucumber Microspores

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Abstract

Direct embryogenesis from cucumber microspores offers the possibilities of highly efficient inbred production and a unique model system for mitochondrial genetics; however, a reliable method of microspore embryogenesis is lacking. We evaluated previously published and new experimental approaches for microspore embryogenesis of cucumber. Plants must be grown in highly controlled environmental conditions within a narrow temperature range to synchronize microspore development. Buds between 5 and 7 mm had a majority of microspores in the late uninucleate stage of development. Isolated buds were treated by cold and heat prior to microspore isolation, and isolated microspores were cultured in the presence of a histone deacetylase inhibitor. Cultured microspores showed changes in the sizes and shapes, potentially an indication of sporophytic development, but did not develop further. A maltose-based culture medium was found to extend the vitality of cucumber microspores in culture. These results and observations should be of interest to researchers trying to develop microspore embryogenesis in cucumber.

Introduction

In vitro culturing and embryogenesis of cucumber microspores offer the possibility to develop large numbers of doubled haploids (DH) for genetic studies and hybrid production. Presently cucumber haploids are produced by culturing female flowers, which is labor intensive with relatively low efficiency at 7 to 11 embryos per 100 ovaries plated (Gałązka et al., 2013). Male flower buds of cucumber produce thousands of microspores and even at a relatively low rate of multicellular development reported at 0.54% to 16.5% could generate hundreds to thousands of embryos (Chen et al. 2018). In comparison, the use of irradiated pollen with embryo rescue typically resulted in 1 to 16 embryos per fruit (Gałązka et al., 2013). Anther culture yielded 1.9 to 2.6 embryos per anther (Gałązka et al., 2013), and even though anthers are more numerous and easier to harvest than ovaries or fruits, this approach yielded fewer embryos per anther than could potentially be achieved with microspores. Moreover, reports of successfully development of cucumber haploids from anther culture may have been insufficiently scrutinized for contaminating sporophytic tissue that may have inflated the success rate (Asadi et al., 2018).

Microspore embryogenesis would also have great value as a tool for transformation of the mitochondrial genome (Havey et al., 2002). Transformation of the organellar genomes would be useful for basic and applied research, for example allowing for the rapid development of novel cytoplasmic male sterile lines for breeding and hybrid seed production, protein production, and stress tolerance (Havey et al., 2002; Laluk et al., 2011). Cucumber microspores offer unique advantages for mitochondrial transformation such as relatively few, large mitochondria that provide larger targets for ballistic particles and paternal transmission of mitochondria to progenies (Havey et al., 2002).

In vitro culture of cucumber microspores has been reported by three research groups. Suprunova and Shmykova (2008) harvested flower buds longer than 6 mm with microspores in the late uninucleate stage and gently macerated the buds in NLN medium supplemented with 10% sucrose. Microspores were filtered through a nylon screen and washed three times with NLN medium followed by centrifugation at 100 xg for 3 minutes. Microspores were cultured in the dark at 22° C for two to four weeks in NLN medium supplemented with 10% sucrose and 2.0 ppm 2,4-dichlorophenoxyacetic acid (2,4-D). Only callus tissue was observed with no further development. Zhan et al. (2009) isolated microspores at the late uninucleate stage from buds that had been pretreated for 2 to 4 days at 4° C. Buds were macerated in B5 medium supplemented with 13% sucrose, the liquid was filtered through a nylon screen, and washed three times with B5 medium with centrifugations at 600 rpm for 3 minutes. The microspores were resuspended at a density of 1 x 10^5 cells/ml in a 2 ml volume of NLN medium with 0.5 ppm 2,4-D and 0.2 ppm 6-benzyladenine (BA) and plated on a 60 mm petri dish. After two days of culture in the dark, some cells were observed to darken and enlarge. After two more

days of culture, cell division was observed and after more than 20 days in culture, plantlets were obtained. Heat treatment of isolated microspores at 33° C for two days did not increase embryogenesis. Finally, Chen et al. (2018) described numerous approaches for microspore embryogenesis in a patent application with apparently high degrees of success. Buds were harvested between 3 and 6 mm at the late uninucleate stage of development. Whole plants were treated at 4° C to 16° C in a growth chamber for one day before harvesting buds, or harvested buds were treated at 0° C to 8° C for one to four days before isolating microspores. Buds were macerated in M404 medium supplemented with 90 g/L of maltose. The liquid was filtered through a 100 micron mesh and then through a 65 to 80 micron mesh. Microspores were washed and spun again for three repetitions and then resuspended at a density of 30,000 to 150,000 cells/ml in a total volume of 4 to 6 ml and cultured in a 60 x 50 mm dish. This final culture medium was M404 supplemented with 3% to 17% w/v of either sucrose or maltose, 0.05 ppm to 2.0 ppm of BA, 0.05 ppm to 2.0 ppm of 2,4-D, 0.5 ppm to 50.0 ppm of phenylacetic acid and 0.5 μM to 40 μM of suberoylanilide hydroxamic acid (SAHA) or 0.001 μM to 1.0 μM of trichostatin A (TSA), although Tables 6 and 9 of the patent strongly suggested that the primary medium used contained 0.5 ppm 2,4-D, 0.2 ppm BA, and 10 μM SAHA. Cultures were subjected to a heat shock of 28° to 35° for 24 to 72 hours and then cultured in the dark at 25° C. Putrescine was added at day seven to promote embryo development. Globular embryos were observed at 20 to 40 days and plantlets were produced shortly thereafter. Numerous accessions were tested and all produced embryos at rates varying from 0.54% to 17.3% of microspores.

Although these studies appear promising for microspore embryogenesis of cucumber, there are no reports of experimental replication of the approaches. The initial report by Suprunova and Shmykova (2008) was found in a conference poster and never published, to our knowledge, in a peer-reviewed journal. The research published by Zhan et al. (2009) has not been repeated by other researchers. Finally, the patent application on cucumber microspore embryogenesis by Chen et al. (2018) has ambiguous language regarding specific details of their protocol. To better understand progress towards embryogenesis of cucumber microspores, new experiments are needed to replicate previous research and to further refine techniques to maximize successful application of this important technology.

Materials and Methods

Cucumber seeds were germinated in vermiculite on a hot pad at 27° C, seedlings were transplanted to a four-inch pot, and then transplanted again when true leaves were present into #1200 11-liter pots in Sungro medium. Temperatures were between 21° C and 24° C during the day and 16° C to 17° C at night with a 16-hour days with high intensity lighting. A closed heating and cooling system maintained environmental conditions. Temperatures were continuously recorded at bench level with a sun shaded probe using an Elitech Temperature and Humidity Data Logger. Several measurements at different times of day were taken with an Apogee Quantum Flux light meter (Model MQ-200) that measured light levels between 200 and 900 μmol/m^2/s. Plants were fertilized every other day at a concentration of 400 ppm of elemental nitrogen (Peters Professional 20-10-20) containing the following micronutrients: 0.15% water soluble magnesium, 0.025% boron, 0.025% chelated copper, 0.1% chelated iron, 0.05% chelated manganese, 0.01% molybdenum, and 0.05% chelated zinc. Deionized water was used for mixing the fertilizer and for watering. Plants were five to eight weeks old when buds were harvested for microspores. Buds from four-week-old plants were isolated on several occasions but microspore production was unacceptably low. No powdery mildew growth was observed at any time on plants.

Three cucumber accessions were used for microspore isolation. USDA Plant Introduction (PI) 518848 was selected because it showed the highest embryogenesis in the patent of Chen et al. (2018). 'Poinsett 76' was also selected because it is closely related to the 'Pointsett 97' reported to be highly embryogenic by Zhan et al. (2009) and moderately embryogenic by Chen et al. (2018). Finally, inbred B was selected from the Polish cultivar 'Borszczagowski' and is highly amenable for plant regeneration from cell cultures (Burza and Malepszy, 1995). Male flower buds were harvested, carefully sized using a digital caliper, and placed on a moist towel until counting and selection was completed (Fig. 1). Cold treatments of harvested buds were in a sealed tube or petri dish with a moistened towel added for humidity. Buds were surface sterilized by treating with 70% ethanol for 30 seconds followed by two washes in sterile water. Buds were then treated with freshly made 1% sodium hypochlorite with 0.01% Tween20 for 10 minutes followed by four washes in sterile water. There was then one last wash in a wash and grinding buffer consisting of Murashige & Skoog modified basal medium with Gamborg vitamins (M404 medium, Phytotechnology Laboratories, Shawnee Mission, KS) supplemented with 10% w/v D-maltose at pH of 5.8. Surface sterilization and washes were conducted in 50 ml conical tubes within a transfer cabinet. Buds were then ground in 0.5 ml of the wash & grinding buffer in a 30 ml sterile glass beaker with a sterile glass stirring rod. Buds were gently but thoroughly macerated to release microspores, and

examination under a microscope showed that the vast majority of microspores were intact (Fig.2).

Liquid from tubes was removed using a pipette tip leaving behind fiber and tissue, and then filtered through a sterile 100 µm cell strainer. The liquid was centrifuged 100 x g for 3 minutes in multiple 1.5 ml tubes. After this first centrifugation, tubes were gently tapped to help collect together microspres that were spread along the side of the tube, and then the liquid was centrifuged again at 100 x g for 3 minutes. The supernatant and any green debris were removed with a pipette and the white pellet was resuspended in 0.5 ml of wash and grinding buffer. Two more washes were conducted to remove as much of any green material as possible leaving a white pellet and a clear supernatant. After the three washes, the pellet was resuspended in M404 medium containing 13% w/v D-maltose, 0.5 ppm 2,4-D, 0.2 ppm BA, and 10 µM SAHA at pH of 5.8. Microspore density was adjusted to 30,000 to 150,000 per ml. Two to five mL of resuspended cells were placed in sterile glass jars (approximately 5 cm in diameter and 6.5 cm in height with a volume of ~130 mL) and sealed with parafilm to prevent evaporation. Jars were placed in the dark at 25° C.

SAHA (commercial name 'Vorinostat' (Selleckchem, Houston, TX)) is potentially useful for reprogramming of microspores from gametophytic to sporophytic development, and was prepared at a concentration of 10 mM in DMSO. Because of the relatively short half-life of SAHA in water, it was added to the medium less than a half hour before the cells were added. To avoid multiple freeze-thaw cycles, the SAHA was aliquoted into small tubes, stored at -20° C, and discarded after each thawing. All other medium components were purchased from PhytoTechnology Laboratories (Shawnee Mission, KS).

Numerous combinations of medium components and concentrations were tested. Filter sterilized maltose and sucrose were tested at 10% and 13% (w/v) in the culture (Suprunova and Shmykova, 2008; Zhan et al., 2009; Scott et al. 1995). Sucrose was also tested as an autoclaved carbon source, which can break down sucrose to produce different composition of sugars. A continuous 25 rpm agitation of isolated microspores in the culture medium was tested based on the reports of improvements in microspore culture with agitation by Yang et al. (2013). Three different culture vessels were tested – 35 mm by 10 mm plastic Falcon Easy Grip petri dish, 100 mm by 15 mm plastic Fisher petri dish, and 50 mm by 65 mm glass jars, based on research by Park et al. (2009) that showed improved embryogenesis of pepper microspores with the utilization of a larger culture vessel, and research by Hoekstra et al. (1992) that showed improvements to microspore culture from exogenous oxygen addition to the culture vessel suggesting that aeration may be important for microspore culture. Different bud treatments were attempted, including 4° C for one to three days and 8° C for one to three days based on a range of temperatures suggested by Chen et al. (2018). Heat treatments of isolated microspores at 30° C for one to three days and 31° C, 32° C, and 33° C were evaluated based on Chen et al. (2018).

Microspores were stained with 1% acetocarmine or 0.01% methylene blue to reveal vacuole development and position of the nucleus. Acetocarmine stains were observed immediately with no washes. Methylene blue stains were observed after incubation for 10 minutes and three washes with M404 medium containing 10% w/v maltose to reduce background staining. Vitality of microspores was estimated using tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), fluorescein diacetate, propidium iodide, DAPI, and a custom stain from Peterson et al. (2010) developed as an alternative to Alexander's stain. The most effective vitality stain was 0.8% tetrazolium dye MTT, which is converted by native enzymes in the cell into dark purple formazan, thus indicating vitality. The MTT stain was diluted to 0.8% in a 5% sucrose solution. All vitality experiments were conducted with MTT. Fluorescein diacetate, propidium iodide, DAPI, and the custom stain were also tested, but not utilized extensively.

Microbial contamination of microspores was assayed by streaking culture liquid onto LB plates containing 1.5% agar and 3% glucose at pH 6.8. Strain LE392 (Promega, Fitchburg WI) of *E. coli* and common baker's yeast were used as positive controls for microbial growth on plates.

Results

Environmental conditions were essential for synchronized production of viable microspores, and strict maintenance of temperatures between 21° C and 24° C during the day and 16° C to 17° C at night with a 16-hour day with high intensity lighting was necessary. Buds from 3 to 6 mm in length were initially selected based on the optimal size for microspores in the late uninucleate stage reported by Chen et al. (2018), although this contradicted Suprunova and Shmykova (2008) who chose buds larger than 6 mm for maximizing late uninucleate stage microspores. Buds in the 3 to 4 mm range had large numbers of tetrads and were discarded. The optimal bud size was 5 to 7 mm which contained mostly vacuolated microspores roughly 50 µm in diameter, and was used for all further experiments.

Cold treatments of isolated buds or entire plants at temperatures ranging from 0° C to 8° C for four days were tested and no cell division or embryogenesis of isolated microspores was observed. Heat shock of the isolated microspores at 28° to 35° for 24 to 72 hours produced no evidence of cell division or embryos.

After one to three days in the dark at 25° C in the M404 medium containing 10% w/v maltose, 0.5 ppm 2,4-D, and 0.2 ppm BA, up to 20% of microspores were observed to enlarge from approximately 40 to 50 microns in diameter, darken in color, and develop a more rounded shape (Fig. 3). These morphological changes and positive MTT staining indicated that the microspores were likely viable after isolation. Plating of the culture medium containing microspores onto LB plates revealed no contaminating microbes.

Despite these cellular changes, no evidence of cell division was observed. Modifications to the protocol included 10% sucrose, 13% sucrose, 10% autoclaved sucrose, 25 rpm agitation, and larger vessel size with a greater trapped air volume, all of which did not result in cell division or embryogenesis. Pretreatments of harvested bud included 4° C or 8° C for one, two, or three days before isolating microspores, and did not induce cell division or embryogenesis. Similarly, heat treatments of the freshly isolated microspores varied from 30° C to 33° C and lasted one to three days, but also resulted in no cell division or embryogenesis.

To determine if the carbon source has an effect on vitality, even if it did not induce cell division, microspores were isolated from 7-week old plants without heat shock and then were divided into either a 10% w/v maltose culture medium or a 10% w/v sucrose culture medium. Microspores were stained with 0.8% MTT on days 0, 1, 2, and 3 after isolation and observed at 40x magnification. MTT staining was scored for several hundred microspores from each time point. In the maltose medium, microspore vitality fell from 15% on day 0 to 7.3% on day 1, 2% on day 2, and less than 1% on day 3. In the sucrose medium, microspore vitality fell from 13% on day 0 to 4.6% on day 1 and no living cells were detectable by MTT staining on day 2.

Discussion

The morphological changes observed for some microspores appear to be consistent with a switch from gametophytic to sporophytic development. Previous researchers have observed that sporophytic development of cucumber microspores appears to show a darkening of the cytoplasm and enlargement of the microspores with a more rounded shape (Suprunova and Shmykova, 2008). Other research has shown that microspores will expand and darken after two day in culture and the vacuoles will disappear in embryogenic microspores (Zhan et al., 2009). Microspores from *Brassica napus* have been carefully observed during sporophytic development and show enlarged size and cytoplasmic changes (Telmer et al., 1993). Maturation of microspores to pollen will also result in enlargement, although no divisions into vegetative and generative cells was observed and at no point did an opaque exine form (Dupuis and Pace, 1993; Telmer et al., 1993). For numerous plants, microspore embryogenesis requires stress treatments or depleted cultures, and non-stressful, enriched conditions lead to pollen development (Dupuis and Pace, 1993; Touraev et al., 1996).

The original experiments on cucumber microspore embryogenesis utilized sucrose as a carbon source (Suprunova and Shmykova, 2008; Zhan et al., 2009). Chen et al. (2018) did not clearly state the carbon source utilized but suggested a broad range of concentrations of either sucrose or maltose. The evidence presented here indicates that maltose is superior to sucrose for maintaining the vitality of cells and presumably for inducing embryogenesis. The rapid death of microspores in a medium with sucrose may be due to hypoxic conditions and ethanol production, both of which are lethal to microspores. Maltose is metabolized more slowly and may avoid hypoxic conditions and resulting in less ethanol production with a more stable osmotic balance (Scott et al., 1995).

In comparing previous experiments on cucumber microspore embryogenesis to these experiments, all the main parameters were tested but did not yield the expected result. Previous experiments had apparent success with 10% sucrose, 13% sucrose, and a range of values from 3% to 17% of sucrose or maltose (Suprunova and Shmykova, 2008; Zhan et al., 2009; Chen et al., 2018). Carbon sources of 10% sucrose, 13% sucrose, and 10% maltose were tested and yielded no embryos. Previous experiments had apparent success with either 2.0 ppm 2,4-D or 0.5 ppm 2,4-D and 0.2 ppm BA (Suprunova and Shmykova, 2008; Zhan et al., 2009; Chen et al., 2018). Plant growth regulators at 0.5 ppm 2,4-D and 0.2 ppm BA were tested and yielded no cell division or embryos. Previous experiments had utilized cold treatments of one to four days of either the buds or the entire plant at temperatures ranging from 0° C to 12° C, although most experiments were conducted at 4° C (Zhan et al., 2009; Chen et al., 2018). Cold treatments of both the buds and whole plants were tested at 4° C and 8° C with no cell division or embryogenesis. Heat shock treatments of 28° to 35° for 24 to 72 hours of the isolated microspores were reported by previous researchers (Zhan et al., 2009; Chen et al., 2018). Heat shock treatments of 28° to 35° for 24 to 72 hours were tested and yielded no cell division or embryos. One previous researcher utilized 10 μM SAHA, a histone deacetylase inhibitor, as a key reagent for reprogramming of microspores to the sporophytic path (Chen et al., 2018). Ten μM SAHA was utilized and resulted in no cell division or embryogenesis. In conclusion, we were not able to repeat the results of previous researchers who reported successful embryogenesis of cucumber microspores, and hope that our observations will be useful for future researchers pursuing this area of research.

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Figure 1. Buds with 5 mm size; bracket indicates where measurement was taken.

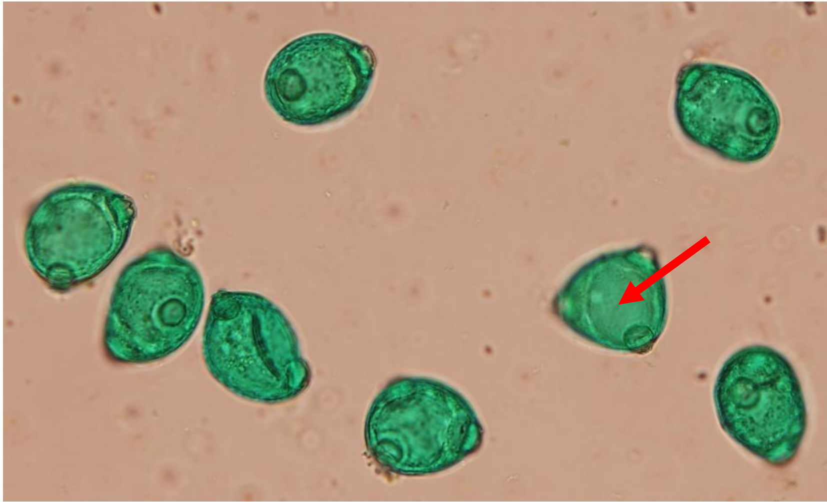

Figure 2. Harvested microspores stained with 1% methylene blue at 400x magnification. Arrow indicates a vacuole typically seen at the late uninucleate stage.

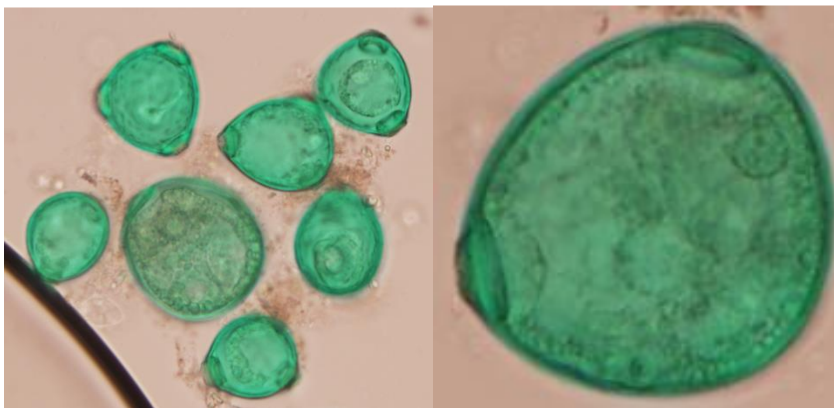

Figure 3. Methylene blue stained microspores 4 days post isolation at 400x magnification. Shown on the left are seven microspores with one darkened and enlarged microspore in the center of the image. Shown on the right is increased magnification of the darkened and enlarged microspore.