Kiwiberry (Actinidia Arguta) Hexaploid Plant Regeneration Through Culture of Endosperm Isolated From Fresh and Year-old Dry Seeds

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Abstract

Endosperm, an ephemeral and storage tissue, serves as a source of nutrition and protection during embryo development and germination. It can be used for the cultivation of polyploid plants *in vitro*. Here, a protocol of plant regeneration and acclimatization from the endosperm-derived calli of *Actinidia arguta* has been developed. Seeds excised from fresh fruit and dry seeds stored for one year served as the sources of endosperm explants of selected tetraploid cultivars of *A. arguta*. Callus Induction Medium (CIM; containing 0.25, 0.5, or 1 mg/l of TDZ) and Actinidia Endosperm Medium (AEM; containing 2 mg/l of 2,4-D and 5 mg/l of kinetin) were used to study the organogenic responses of the calli. On AEM, the source of explant did not significantly affect the rate of callus induction for any of the tested cultivars. Similarly, no organogenic events were observed. In contrast, on CIM both the source of explants and the cultivar origin caused significant differences in callus formation and subsequent organogenic events. Histological and ultrastructural analyses revealed the adventitious nature of shoot bud formation on these media. The most efficient elongation of shoot buds was achieved after transferring organogenic calli with adventitious shoot buds to a medium supplemented with zeatin or *meta*-topolin. Robust root induction with minimal basal callus formation occurred on the medium with indole-3-acetic acid. Flow cytometric analysis revealed that the nuclear DNA content in the leaves of some regenerants (4.5 pg/2C) was approximately 50% higher than that in the tetraploid seedlings (3.1 pg). This finding confirmed that those regenerants originated from the endosperm. The regeneration of hexaploid plants was more efficient when endosperm from fresh seeds served as an explant; therefore, fresh rather than dry seeds are recommended for endosperm-derived plant production. The hexaploid plants of *A. arguta* can serve as an important source of breeding material.

Key Message

This is the first study to reveal that the genotype, media composition, source, and age of explants significantly affected the induction of endosperm-derived callus and subsequent plant regeneration in kiwiberry.

Introduction

Due to their origin, endosperm cells usually possess a higher nuclear DNA content (3C) than the embryo (2C), which results from the doubled fertilization that occurs in flowering plants. Additionally, endosperm also plays a very important role during seed development and germination, acting as a vital repository of nutrients for the developing and germinating embryo (Kumar and Gupta 2015).The relationship between ploidy level and some fruit characteristics (e.g., fruit weight or flesh color) is crucial for the breeding and trade of kiwifruit (Li et al. 2010; Wu et al. 2012). To obtain polyploid plants *via* conventional methods, long and labor-intensive procedures are usually required. Plant tissue culture techniques can provide a relatively faster alternative for obtaining polyploid plants (Wang et al. 2016). One of such technique is culturing of isolated endosperm tissue followed by plant regeneration. To date, nearly 70 species have been cultured under *in vitro* conditions using endosperm cultures. However, the ability of isolated endosperm tissue to proliferate and for plants to regenerate has been found for fewer than 40 of the species (Hoshino et al. 2011; Wang et al. 2016). Furthermore, the plant growth regulators (PGRs) added to the culture medium, the sources of the explant material, and the cultivar of the donor plants all play an important role in establishing a successful regeneration protocol from endosperm explants (Wang et al. 2016). Despite being challenging work, the recently published protocols for endosperm-derived plant regeneration in *Gomortega keule* (Muñoz-Concha 2016), *Melia azedazach* (Thang et al. 2018), *Passiflora edulis* (Antoniazzi et al. 2018), and *Passiflora cincinnata* (Silva et al. 2020) indicate that this technique is still attracting interest and is worth applying to economically important plant species.

The native distribution range of the genus *Actinidia* (with 54 species and more than 100 taxa) is Asia, especially southwestern China (Ferguson and Huang, 2007; Ferguson 2016; Huang 2016). Most of these species, however, are cold-sensitive. Only selected species, such as *Actinida arguta* (Siebold et Zucc.) Planch. ex Miq., known as kiwiberry or sometimes as “cold-hardy kiwifruit” or “mini-kiwi,” can be cultivated in regions where temperatures can fall even below -30 °C (Debersaques et al. 2015). In addition to cold resistance, this species features grape-sized, hairless fruits which at harvest stage are delicate, aromatic, and rich in vitamin C, carotenoids, and polyphenols (Latocha 2017). On the global fresh fruit market, the kiwiberry is less common than the more popular “fuzzy” kiwifruit, *A. chinensis* var. *deliciosa* (Cossio et al. 2015; Melo et al. 2017). However, a positive trend has been noticed towards the establishment of new kiwiberry plantations in countries such as Poland, Belgium, and the Netherlands due to the fact that more and more people are becoming aware of the health benefits associated with kiwiberry consumption (Debersaques et al. 2015).

In the genus *Actinidia*, successful plant regeneration through cultured endosperm has been reported for *A. chinensis* (Gui et al. 1982), the hybrids *A. chinensis* × *A. melanandra* and *A. arguta* × *A. deliciosa* (Mu et al. 1990), *Actinidia* ssp. (Machno and Przywara 1997), *A. chinensis* var. *deliciosa* (Góralski et al. 2005; Chłosta et al. 2021), and *A. kolomikta* (Asakura and Hoshino 2017). However, Machno and

Przywara (1997) found that during the culturing of endosperm-derived callus from an unidentified cultivar of *A. arguta*, only roots and a few abnormal shoots were formed and no normal regenerants suitable for further breeding developed.

The aim of the present study was to develop a protocol for plant regeneration from the isolated endosperm tissue of selected cultivars of *A. arguta*. An experimental system established previously for plant regeneration from endosperm tissue in *A. chinensis* var. *deliciosa* (Góralski et al. 2005) turned out to be somewhat appropriate for *A. arguta*. Since numerous previous studies have demonstrated that the cultivars can differ in the regeneration capacity, four cultivars were tested. Additionally, the study investigated whether the source of the material (seeds excised from fresh fruit versus dry seeds stored for one year) influences proliferation and regeneration potential. The results increase the knowledge of additional plant species with the ability to proliferate *in vitro* through endosperm and to regenerate plants possessing 3C nuclear DNA content and contribution of a double maternal DNA.

Materials And Methods

Plant materials

Vines of kiwiberry (*A. arguta*) were selected from the plant germplasm collection at Warsaw University of Life Sciences (SGGW), located in Warsaw, Poland. Four female cultivars were used in the current study – 'Geneva,' of American origin, 'Weiki,' of German origin, and two Polish cultivars, 'Scarlet September Kiwi'® and 'Bingo'PBR. They all are tetraploids with a chromosome number of 2n = 4x = 116 (Melo et al. 2017). The plants were exposed to open pollination and mature (soft, ready-to-eat) fruits were collected at the end of September in 2018 and 2019. The harvested fruits was divided into two lots. One lot was placed in small, breathable plastic boxes immediately after collecting and was stored at 10 °C in a refrigerator, to be used as the endosperm source for the fresh material (fresh seeds). From the second lot, the pulp and seeds were taken out, washed first in tap water and then in distilled water, dried at room temperature for a few hours, and then stored for one year at room temperature in a glass jar; they were used as the endosperm source for the dry material (dried seeds). The fruits was sterilized, the seeds dissected from the fruit, the seed coats excised, the embryos isolated, and the endosperm tissue dissected (Fig. 1a) according to a protocol previously developed for *A. chinensis* var. *deliciosa* (Góralski et al. 2005). The dried seeds after the sterilization were soaked in distilled, sterile water for 24 h at room temperature.

Callus induction

The endosperms isolated from fresh seeds and the one-year-old dry seeds were inoculated on 60-mm Petri dishes with a culture medium based on the Murashige and Skoog (1962) (MS) macro-, microelements, and vitamins (Duchefa), supplemented with 3% sucrose and 0.6% agar (Duchefa) in addition to the PGRs. For the callus induction medium (CIM), the medium described above was supplemented with 0.25 (CIM-0.25), 0.5 (CIM-0.5), or 1.0 mg/l (CIM-1.0) of thidiazuron (TDZ) and Actinidia Endosperm Medium (AEM) with 2 mg/l of 2,4-dichlorophenoxy-acetic acid (2,4-D) and 5 mg/l of kinetin (KIN). CIM without any PGRs (CIM-0) was used as a control medium. Sealed with Parafilm®, the Petri dishes with endosperm explants were incubated at 25 °C in the dark. The proliferating explants were transferred to fresh medium every three weeks and incubated under the same conditions.

Observations and images were performed using a dissecting binocular microscope (Zeiss Stemi SV 11, Germany) that was equipped with a digital camera (Canon Power Shot G6). The images were processed with CorelDRAW Graphics Suite 2020 software.

Shoot bud development

Calli with visible (with a height of approx. 5 mm) adventitious shoot primordia were transferred to Petri dishes 90 mm in diameter and 25 mm in height (Phoenix Biomedical) containing six types of Shoot Development Medium (SDM). All SDMs contained full-strength MS salts and vitamins (Duchefa), 3% sucrose, and 0.6% agar. SDM0 did not contain any PGRs, while SDM1 was supplemented with 0.5 mg/l of TDZ, SDM2 with 2 mg/l of 6-Benzlyaminopurine (BAP), SDM3 with 2 mg/l of kinetin (KIN), SDM4 with 2 mg/l of zeatin (ZEA), and SDM5 with 2 mg/l of *meta*-topolin (*m*T). All cultures were incubated at 25 °C with a 16-h photoperiod under cool-white fluorescent tubes (60–90 µmol $m^{-2}s^{-1}$) for 60 days.

Rooting and acclimatization

Regenerated shoots with a height of approx. 2–3 cm were excised from the callus clumps. Micro-cuttings were sub-cultured in Magenta™ vessels (Sigma) with four types of Root Induction Media (RIM). All RIMs were provided with half-strength MS salts and vitamins (Duchefa), 2% sucrose, and 0.6% agar (Plant Agar, Duchefa). RIM0 did not contain any PGRs, while RIM1 was supplemented with 0.2 mg/l of indole-3-acetic acid (IAA), RIM2 with 0.2 mg/l of indole-3-butyric acid (IBA), and RIM3 with 0.2 mg/l of 1-napthaleneacetic acid (NAA). The cultures were kept under the same temperature and photoperiod conditions as described above. Shoots with a well-developed root system were

removed from the culture vessels and gently washed in a beaker with sterilized distilled water to wash out the RIM. Afterwards, the plants were placed in small pots with commercial substrate for seeding (Substral). The potted plants were watered and covered with a plastic bag to maintain high relative humidity. After two weeks in humid conditions, holes were made in the plastic bags in order to gradually reduce the humidity. Two months later the shoots were transferred from pots to garden soil conditions in the middle of August 2019. The plants were grown under green netting to protect them from direct sunlight.

Light and scanning electron microscopy

Six-week-old intact calli with shoot bud domains were collected from CIM-0.5TDZ and fixed overnight at 4°C in a solution of 5% (v/v) glutaraldehyde in 0.1 M phosphate buffered saline (pH: 7.2). Next, the samples were washed four times in phosphate buffer and dehydrated gradually in an ethanol series, from 10% to 100% (v/v). The basal stem segments from *in vitro* rooting shoots with 10-day-old root primordia were excised and fixed as described above. The fixed tissues were embedded in Technovit® 7100 synthetic resin and 5-µm-thick sections were cut, stained with 0.1% (w/v) toluidine blue, and finally mounted in Entellan (Merck, Darmstadt, Germany) according to the procedure described by Popielarska et al. (2006). Observations and documentation were performed using the visible light system of a Nikon Eclipse E400 microscope equipped with a Zeiss AxioCam MRe digital camera and Zeiss AxioVision 3.0 software and a Nikon DS-Fi2 with NIS-Elements 4.0 software.

For scanning electron microscope (SEM) analysis, the fixed samples, after dehydration through a graded ethanol series, were dried with CO_2 critical-point drying and coated with gold (Jeol JFC-1100 E ion-sputtering system). Observations were made with an SEM (HITACHI S-4700).

Flow cytometry

Flow cytometric (FCM) analysis was performed on leaf samples from 35 rooted regenerated plants obtained from the endosperm-derived callus of 'Bingo' seeds from both sources of explants – fresh seeds (15 regenerants) and year-old dry seeds (20 regenerants). Leaves from five seedlings of the tetraploid cultivar 'Bingo' grown *in vitro* were used as a ploidy control. The leaf samples were prepared as previously described (Sliwinska and Thiem 2007) using nuclei isolation buffer (200 mM TRIS, 4 mM $MgCl_2 \cdot 6H_2O$, 0.5% [v/v] Triton X-100; pH=7.5) supplemented with propidium iodide (50 µg/mL) and ribonuclease A (50 µg/mL). *Solanum lycopersicum* (1.96 pg/2C; Doležel et al. 1992) served as an internal standard. The nuclear DNA content was estimated using a CyFlow SL Green (Partec GmbH, Münster, Germany) flow cytometer equipped with a high-grade solid-state laser with green light emission at 532 nm, a long-pass filter RG 590 E, DM 560 A, as well as side and forward scatters. The nuclear DNA content was calculated using the linear relationship between the ratio of the 2C peak positions of *Actinidia*/*Solanum* on a histogram of fluorescence intensities. For each sample, the nuclear DNA content in 5000–8000 nuclei was measured by applying linear amplification. Histograms were evaluated using the FlowMax (Partec GmbH, Münster, Germany) program. The coefficient of variation of the G_0/G_1 peak of the *Actinidia* species ranged from 2.43% to 4.91%.

The Cx value was calculated based on the genome size of the control tetraploid plants and was applied to establish a DNA ploidy of regenerants. Cx refers to the DNA content of a monoploid genome with the chromosome base number x (Greilhuber et al. 2005). Thus, the abbreviation 4Cx is used for the 2C DNA content of a tetraploid plant and 6Cx for that of a hexaploid. However, it should be noted that hexaploid plants originated from endosperm possessing 3C DNA content, as compared to the source tetraploid plants.

Data recording and statistical analysis

Statistical analysis was performed with the use of R software v. 3.6 (R Core Team 2020). Confidence intervals (confidence level: 0.95) for proportions were calculated with proportion test (*prop.test*() function) and the proportions were compared with a use of Fisher's Exact Test for Count Data (*fisher.test*()) to examine the effects of the cultivar, the medium, and the source of explant on callus proliferation and shoot bud regeneration; differences were regarded as significant when p was less than 0.05.

Almost 3000 explants were used for this study of endosperm response (Table 1). For each kind of medium, cultivar, and source of explant, observations were made to record the proportion of calli and shoot bud induction (for establishing the efficiency of callus and shoot bud induction). The explants' response was recorded at four-week (for callus induction) and eight-week (for establishing shoot buds induction) intervals after inoculation.

The experiments were carried out in 1–3 trials and with different numbers of explants.

Results

Callus and shoot bud induction

Callus induction was observed for all of the tested cultivars, for both explant sources, and for all tested media (apart from the control one). The first proliferation and enlarging of explants were visible after 7–12 days of culturing (Fig. 1b, c). The callus growing on the AEM was soft and aqueous in appearance, while the callus from the CIMs was semi-compact and a light yellow, creamy color. During the subsequent days of culturing, the size of the calli cultured on the AEM increased and no organogenic events were observed. Adventitious shoot primordia were visible as light green bulges (Fig. 1d, e). They were observed on all CIMs after approx. 42 days for 'Bingo' and on CIM-0.5 after 60 and 80 days for 'Scarlet September Kiwi' and 'Geneva', respectively. Only for 'Weiki' was no formation of shoot primordia observed on CIM-0.5.

The source of explant (fresh versus year-old seeds) did not significantly affect callus induction on AEM for any cultivar (Table 1). However, on CIM-0.5 the efficiency of callus formation on the fresh seeds' endosperm was significantly higher for 'Geneva' (the proportions of the number of calli to the number of explants were 0.92 and 0.75 for fresh and year-old seeds, respectively) and 'Scarlet September Kiwi' (0.89 vs. 0.42). Explants obtained from fresh 'Weiki' seeds exhibited the lowest efficiency (0.35) of callus formation on CIM-0.5. There were no significant differences in the number of calli induced per explant between cultivars for the same source of explant on AEM, but such differences were found on CIM-0.5. For fresh seeds, more calli were observed on 'Geneva' and 'Scarlet September Kiwi' endosperms (0.92 and 0.89, respectively) than in the case of 'Bingo' (0.83). Explants derived from year-old 'Bingo' and 'Geneva' seeds generated more calli (0.79 and 0.75, respectively) than from 'Scarlet September Kiwi' (0.42).

'Bingo' was the only cultivar for which all media were tested, but only for fresh seeds. The efficiency of callus induction (no. calli/no. explants) was similar among three media – on CIM-1.0 it was 0.99, on AEM 0.97, and on CIM-0.25 0.95 – and was significantly higher than on CIM-0.5 (0.83). The results for year-old seeds were similar, reaching 0.89 and 0.79 on AEM and CIM-0.5, respectively.

The explants of 'Geneva' and 'Scarlet September Kiwi' were tested only on AEM and CIM-0.5. The only significant difference found in callus induction efficiency in a given explant class and cultivar was for explants from year-old 'Scarlet September Kiwi' seeds: on AEM more explants induced calli (0.78) than on CIM-0.5 (0.42).

As mentioned above, buds were formed on calli only when CIMs were used. On CIM-0.5, fresh-seed-derived 'Bingo' explants reached approx. 10 and 20 times higher efficiency of bud formation (0.22) – calculated as the number of calli with buds divided by the number of explants – than 'Scarlet September Kiwi' (0.02) and 'Geneva' (0.01). Also, when the proportion of calli with buds to the total number of calli was analyzed, 'Bingo' demonstrated similar superiority. The calli from year-old seed endosperm only generated buds in the case of 'Bingo' (0.11), with significantly less efficiency than for fresh seed explants (Table 1).

Because of the 'Bingo' cultivar's high efficiency in bud formation per explant on CIM-0.5, its fresh explants were also tested on other CIMs. The results were similar for CIM-0.25 (0.20), but significantly lower for CIM-1.0 (0.06).

Plant regeneration

The transfer of calli with adventitious shoot buds on SDM1, SDM2, and SDM3 resulted in limited growth of shoots. Initially, the calli turned into compact, green clumps, but during the following weeks all explants with shoot buds became necrotic. Successful shoot elongation was obtained on SDM4 and SDM5 in the case of 'Bingo' and on SDM4 in the case of 'Scarlet September Kiwi' (Fig. 1f). It is important to mention that we were only able to test different SDMs for shoot elongation with 'Bingo' because only this cultivar produced a sufficient number of calli clumps with shoot primordia. Due to the limited number of calli with shoot primordia in 'Scarlet September Kiwi' and 'Geneva' from fresh seeds (Table 1), we decided to use SDM4 for shoot elongation in these cultivars, based on our prior promising results with 'Bingo'. However, only endosperm-derived callus with shoot buds obtained on CIM was capable of forming complete shoots when transferred to SDM4 (Fig. 1f); the rates of elongated shoots produced by different cultivars were different (Table 2). During the elongation and development of shoots on SDM4 and SDM5, an intense reddish/pinkish pigment formation was noted in the callus, mostly confined to the region where shoot buds were growing. Shoots 1 to 3 cm in height were obtained after 60 days of culturing on SDM4 and SDM5 (Fig. 1f). On SDM0, the callus tissue with adventitious shoot buds started turning black after three days of subculturing and ultimately turned completely necrotic after seven days of culturing.

After two months of culturing on SDM4 and SDM5, well-developed shoots of 'Bingo' were excised from the growing clumps of the callus (Fig. 1f) and transferred onto different RIMs (Table 3). All treatments resulted in root and callus formation near the basal end of the stem. However, a root system accompanied by a small amount of callus was formed on RIM1 (Fig. 1g) after 30 days of subculturing. Swelling of the basal part of the stem was noted after five days, along with callus induction, followed by a clearly visible formation of the root meristem after ten days of culturing on RIM1 (Fig. 1g, inset). Explants cultured on RIM2 and RIM3 promoted a large basal callus formation

at the basal part of the stem. Excised shoots cultured on RIM0 produced few, slow-growing roots as compared to the RIMs containing auxins.

Acclimatization

Fifty plants with a well-developed *in vitro* root system were acclimatized. During the first week of the acclimatization, the majority of the plantlets started shedding the leaves which had been formed during *in vitro* culturing. However, after a few days, swelling of the nodes was observed and healthy-looking, green leaves started to grow from the nodes. One month of acclimatization resulted in 25 plants (Fig. 1h) ready to be transferred to garden conditions; the survival rate was 50%. Fully acclimatized regenerated plants (Fig. 1i) were obtained within only six months of the endosperm being isolated, which confirms the efficacy of the protocol.

Ultrastructural and histological analyses

The ultrastructural and histological analyses of the endosperm explants (Figs 2a–b1) and six-week-old calli cultured on CIM-0.5 (Fig. 2c, d) were performed using an SEM and a light microscope. Sections of freshly isolated endosperm revealed thick-walled cells with abundant storage substances (Fig. 2a, a1). On sections of endosperm explants after 14 days of culturing, post-division cells without storage substances were detected (Fig. 2b, b1). Histological sections of six-week-old cultures revealed the formation of multiple shoot bud domains (Fig. 2c). These structures were composed of cells with uniform size and shape and dense cytoplasm, covered with an epidermal-like layer, and maintaining continuity with the callus. The formation of shoot buds was also noted using SEM (Fig. 2d); callus domains were surrounded by small, irregular clumps of parenchymatic cells. Cross-sections of stem from the micro-cutting, cultured for 10 days on RIM1, showed little induction of basal callus and lateral root primordia (Fig. 2e). The vascular connection between the xylem of the main stem and the developing root was clearly visible.

Nuclear DNA content and DNA-ploidy

The 2C nuclear DNA content in the control tetraploid plants of ‘Bingo’ was 3.15 pg (the mean for nine individuals) (Fig. 3, Table 4). Twenty-five regenerated plants, regardless of the source material, formed two distinct groups with clearly different genome sizes, one with about 3.1–3.2 pg/2C and the other with 4.5 pg/2C of DNA. Since the latter one (17 regenerants) possessed approx. 1.5 times the amount of DNA of the tetraploid control, they were considered to be hexaploids (6Cx) of endosperm origin. The remaining regenerants possessed DNA content similar to the control, so their DNA ploidy was estimated as tetraploid (4Cx). The frequency of hexaploid regenerants differed depending on the explant source: when endosperm was isolated from fresh seeds, 14 per plantlets were 6Cx, while this proportion among regenerants obtained from the dry seeds decreased to 3 per 20. The monoploid genome size (1Cx) for tetraploids was about 0.79 pg and for hexaploids 0.75 pg.

Discussion

Callus induction and plant regeneration

Endosperm tissue was previously successfully used for the *in vitro* regeneration of species in the genus *Actinidia* (Hoshino et al. 2011). In that study, the endosperm was isolated only from seeds obtained from fresh fruits. The current work is the continuation of studies on isolated endosperm of *A. arguta* ‘Bingo’ (Popielarska-Konieczna et al. 2020), which revealed that cultured explants displayed structural and chemical differences in the cell wall depending on the type of morphogenic pathway. In the present study, we focused on culture medium composition – especially PGRs – the type of cultivars, and the source of explants (isolated from fresh fruits or year-old dry seeds) as important factors in establishing a successful plant regeneration system from the endosperm of *A. arguta*. Changing the concentration of PGRs in the culture medium and applying them individually or in combination can result either in direct or indirect organogenic developmental pathways (Feher 2019). Highly efficient callus induction and proliferation was observed from the isolated endosperm explants (obtained from fresh as well as dry seeds) of *A. arguta* in all tested cultivars on CIM and AEM. However, a morphogenic response occurred only on CIM. TDZ has been reported to be used in the successful regeneration of plants with different ploidy levels *via* endosperm-derived callus (Góralski et al. 2005; Popielarska et al. 2006; Sun et al. 2011; Antoniazzi et al. 2018). TDZ is a phenlyurea compound that exhibits both auxin- and cytokinin-like properties and is more stable to withstand degradation by cytokinin oxidases (Mok et al. 1982). It also increases the accumulation of endogenous cytokinins and the translocation of auxins and it interacts synergistically with other culture media additives which induce shoot bud primordia (Murthy et al. 1998; Vinoth and Ravindhran 2018). Góralski et al. (2005) reported that *A. deliciosa* plants were regenerated from endosperm-derived callus on a medium supplemented with TDZ alone, during an extended period of subculturing. Similarly, TDZ was found to be the only cytokinin among all those tested that induced shoot production in endosperm explants from commercially grown passionfruit (Antoniazzi et al. 2018). On the contrary, in the present study, maintaining calli with

adventitious shoot primordia for a longer period of time on CIMs inhibited elongation and resulted in the formation of highly hyper-hydric leaf-like structures. Our findings regarding the negative effects of TDZ on shoot elongation are in agreement with studies on *Leucaena leucocephala*, in which the authors reported an inverse relationship between TDZ and shoot elongation due to basal callusing, shoot fasciation, and necrosis (Shaik et al. 2009; Pal et al. 2012).

To overcome the problem of shoot elongation, it is recommended to transfer calli with adventitious shoot buds to a medium containing adenine-type cytokinins and auxins (Vinoth and Ravindhram 2018). In *A. arguta*, the microscopic shoot buds successfully elongated to a height of 1–3 cm when transferred from CIM to SDM4 or SDM5. ZEA, the first naturally occurring cytokinin to be identified, has been widely used in studies on plant tissue culture, such as those performed on *Vaccinium* (Reed and Abdelnour-Esquivel 1991), *Olea europea* L. (Farooq et al. 2017), and most recently *Solanum melongena* L. (García-Fortea et al. 2020). ZEA has already proven its organogenic potential in the genus *Actinidia*, in terms of adventitious shoot bud induction and elongation from callus cultures (Mu et al. 1990; Liu et al. 1998; Takahashi et al. 2004; Lan et al. 2007). In *A. kolomikta*, shoot elongation was obtained only when an endosperm-derived callus with adventitious shoot primordia was cultured on ZEA (Asakura and Hoshino 2017). A novel derivative of the family of aromatic cytokinins, *m*T, has revealed promising results in plant organ culture studies (Jameson 2017). The successful use of *m*T for shoot elongation was reported for *Pistachio vera* (Benmahioul et al. 2012; Marin et al. 2016), *Daphne mezereum* 'Alba' (Nawakoska et al. 2019), *Tecoma stans* (Hussain et al. 2019), a red-fleshed kiwifruit cultivar (Saeiahag et al. 2019), and *Allamanda cathartica* (Khanam et al. 2020). In the present study, a greater number of fully developed elongated shoots were obtained on a medium with *m*T than on a medium with ZEA, which is in accordance with previous studies. Moreover, we observed that shoots grown on a medium supplemented with *m*T or ZEA were high-quality and grew vigorously when transferred to RIMs.

Regarding rooting, the micro-cuttings of *A. arguta* responded positively to different RIMs, though very minimal callus formation at the basal end of the stems occurred on a medium supplemented with IAA. On the contrary, robust rooting of micro-cuttings in *A. deliciosa* (Góralski et al. 2005) and *A. kolomikta* (Asakura and Hoshino 2017) was obtained on a half-strength-MS medium that was free of PGRs. The role of auxins, in turn, is well established in *in vitro* rooting of micro-cuttings and those hormones are widely used in plant species known to be recalcitrant to rooting (Fogaca and Fett-Neto 2005). In studies on endosperm culturing of *Carica papaya* (Sun et al. 2011) and *Melia azedazach* (Thang et al. 2018), more efficient rooting was reported when a small amount of auxins was added to the culture medium, which validates the findings of the current work.

The role of the cultivar and the age of explant source

Cultivars plays an important role in establishing a successful plant regeneration system. A prominent cultivar effect was observed in the present study on *A. arguta*, especially in terms of the greater efficiency of explants producing calli with shoot buds in 'Bingo' in comparison to 'Scarlet September Kiwi', "Geneva', and 'Weiki'. Many authors reported a highly cultivar-dependent response of explants cultured *in vitro* in different species (Jach and Przywara 2000; Scalzo et al. 2016; Farooq et al. 2017; Mostafa et al. 2020) . In terms of callus size and subsequent plantlet regeneration of different cultivars of *Vaccinium* spp., 21% of the variation was attributed to cultivar (Scalzo et al. 2016). Also, a strong cultivar effect was observed during the propagation of olive (Farooq et al. 2017) and sunflower cultivars (Jach and Przywara 2000). Similarly, Mostafa et al. (2020) found significant differences between the rate of callus induction among four cultivars of garlic.

In the present study, culturing *A. arguta* endosperm isolated from year-old dry seeds resulted in plant regeneration, which may be of great significance due to the fact that fresh fruits are not available year-round. Similar studies on other species have yielded various results. Zhu et al. (1997) obtained regenerated plantlets from dry mature endosperm cultures of *Eucommia ulmoides*, but for *Gomortega keule*, the attempt to regenerate plantlets from the endosperm isolated from year-old dry seeds failed (Muñoz-Concha 2016). In the case of the genus *Actinidia*, no reports have been published to date on endosperm culturing from dry seeds. Our work demonstrates for the first time that the regeneration of complete plants with 3C DNA nuclear content from explants obtained from year-old dry seeds of *A. arguta* is possible – if not very efficient.

Nuclear DNA content and DNA-ploidy

Chromosome counting is a direct method to establish the ploidy of a plant. This approach can be very laborious and unreliable in *A. arguta* because of small size and relatively high number of chromosomes (>110) (Yan et al. 1997; Huang 2016). On the other hand, FCM is a quick and reliable method of measuring nuclear DNA content in plants and DNA ploidy can be deduced from the 2C value if a control plant of known ploidy is available. The method has been used extensively in estimating the genome size of horticultural and medicinal plants, especially in polyploid and hybrid breeding (Sliwinska 2018). FCM has been used to confirm the endosperm origin of regenerated plants, for example, of *A. chinensis* var. *deliciosa* (Góralski et al. 2005), *Gomortega keule* (Muñoz-Concha 2016), *A. kolomikta* (Asakura and

Hoshino 2017), *Melia azedazach* (Thang et al. 2018), and *Passiflora cincinnata* (Silva et al. 2020). The FCM analysis done in this study on *A. arguta* regenerants from endosperm-derived calli, obtained from both sources of explants, confirmed the hexaploidy (6Cx = 3C DNA content) of the regenerants through a comparison with the tetraploid seedlings of a commercially available tetraploid cultivar. However, a high proportion of desirable 6Cx plants were obtained only when fresh seeds were used as the source of explants. In our experience, isolating endosperm tissue is much easier using seeds from fresh fruits than dry seeds. That in turn makes it more likely, that some cells of the embryo, which adhere closely to the endosperm in dry seeds, can be included in the explant of the latter plant material. The 2C cells usually have a higher totipotency than those of higher ploidy (e.g. Gilissen et al. 1994; Kubaláková et al. 1996), and thus a higher proportion of tetraploid than hexaploid *A. arguta* plants were regenerated in this experiment. For this reason, when using dry seeds as a source of endosperm, estimating the ploidy of the regenerants is strongly recommended. Nevertheless, using endosperm explants from fresh seeds is rather suggested for a successful 6Cx plant regeneration of this species. However, because of the unavailability of fresh fruits of *A. arguta* all the year round, using endosperm explants from stored dry seeds seems to be an alternative.

When comparing the 1Cx values of tetraploid and hexaploid plants (0.79 pg vs. 0.75 pg), a downsizing of the monoploid genome size is observed. This may be a result of aneuploidy (as suggested by Chłosta et al. 2021).

Ultrastructural and histological analyses

Ultrastructural and histological analyses provide an opportunity to better understand and distinguish between different morphogenic events occurring under *in vitro* culture conditions (Haensch 2004; Kurczyńska et al. 2007; Dobrowolska et al. 2017). Most studies have reported plant regeneration *via* shoot bud formation from an endosperm-derived callus; in a few cases, somatic embryogenesis also occurred when endosperm explants were cultured (Hoshino et al. 2011). In *A. arguta*, the SEM and histological analyses of organogenic callus done as part of this study clearly depicted the induction of adventitious shoot buds from the endosperm-derived callus, followed by plant regeneration. The findings of this work are in agreement with previous microscopic observations carried out on an isolated endosperm culture in the *Actinidia* taxon (Góralski et al. 2005; Asakura and Hoshino 2017). In those studies, the dedifferentiation of storage tissue to parenchymatic callus was clearly visible. Other studies have reported the adventitious nature of shoot bud formation in an endosperm-derived callus (Popielarska et al. 2006; Popielarska-Konieczna et al. 2020; Czernicka et al. 2021).

Conclusions

In conclusion, we have demonstrated for the first time that the isolated endosperm of kiwiberry, *A. arguta*, could be successfully used as an explant for callus proliferation. A full protocol of plant regeneration from endosperm and the acclimatization of regenerants has been developed. The type of tetraploid cultivar (2C = 4Cx) and the source of explants (fresh vs. year-old dry seeds) affected the ability to form of regenerants. The genome size of tetraploids of *A. arguta* was established as 3.1 pg/2C and of hexaploids 4.5 pg/2C. Because of the probability of regenerating undesirable tetraploid plants, seeds isolated from fresh fruit than dry seeds should be used as a source of endosperm, if possible. Nevertheless, in all cases the ploidy of the regenerants should be established because of the probability of obtaining 4Cx plants, regenerated from the remnant of the embryo. The present findings are valuable because hexaploid plants can be an important source of breeding material for the further improvement of kiwiberry germplasm. Moreover, future studies using histological and molecular techniques can provide more detailed insights into the organogenic and non-organogenic events of the isolated endosperm tissue.

Declarations

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Author contribution statement

MPK devised the research. MA, MT, and PW performed the tissue cultures. MA and MPK performed the histological and SEM studies. MA and PL visualized the results. ES carried out the flow cytometry analysis and evaluated the results. GG carried out the statistical analysis.

MA, ES, and MPK contributed to the writing of the manuscript. All of the authors read and approved the final manuscript.

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Tables

Table 1. Efficiency of callus and shoot buds induction of isolated endosperm in *Actinidia arguta* 'Bingo', 'Geneva', 'Scarlet September Kiwi', and 'Weiki'.

CI – confidence intervals (confidence level = 0.95), P – proportion.

Medium	Cultivar	Source (seeds)	No. of explants	No. of calli	No. of buds	P of calli per explant	CI-	CI+	P of buds per explant	CI-	CI+	P of buds per calli	CI-	CI+
	'Bingo'	fresh	105	0	0	-	-	-	-	-	-	-	-	-
	'Bingo'	dry	45	0	0	-	-	-	-	-	-	-	-	-
Control	'Geneva'	fresh	60	0	0	-	-	-	-	-	-	-	-	-
	'Geneva'	dry	45	0	0	-	-	-	-	-	-	-	-	-
	Scarlet Sept. Kiwi'	fresh	60	0	0	-	-	-	-	-	-	-	-	-
	Scarlet Sept. Kiwi'	dry	45	0	0	-	-	-	-	-	-	-	-	-
	'Bingo'	fresh	105	102	0	0.97	0.91	0.99	-	-	-	-	-	-
	'Bingo'	dry	45	40	0	0.89	0.75	0.96	-	-	-	-	-	-
AEM	'Geneva'	fresh	60	56	0	0.93	0.83	0.98	-	-	-	-	-	-
	'Geneva'	dry	45	36	0	0.80	0.65	0.90	-	-	-	-	-	-
	'Scarlet Sept. Kiwi'	fresh	60	55	0	0.92	0.81	0.97	-	-	-	-	-	-
	'Scarlet Sept. Kiwi'	dry	45	35	0	0.78	0.63	0.88	-	-	-	-	-	-
CIM-0.25	'Bingo'	fresh	105	100	21	0.95	0.89	0.98	0.20	0.13	0.29	0.21	0.14	0.31
	'Bingo'	fresh	435	359	96	0.83	0.79	0.86	0.22	0.18	0.26	0.27	0.22	0.32
	'Bingo'	dry	105	83	12	0.79	0.70	0.86	0.11	0.06	0.19	0.14	0.08	0.24
CIM-0.5	'Geneva'	fresh	330	303	3	0.92	0.88	0.94	0.01	0.00	0.03	0.01	0.00	0.03
	'Geneva'	dry	105	79	0	0.75	0.66	0.83	-	-	-	-	-	-
	'Scarlet Sept. Kiwi'	fresh	330	295	8	0.89	0.85	0.92	0.02	0.01	0.05	0.03	0.01	0.05
	'Scarlet Sept. Kiwi'	dry	105	44	0	0.42	0.32	0.52	-	-	-	-	-	-
	'Weiki'	fresh	223	77	0	0.35	0.28	0.41	-	-	-	-	-	-
CIM-1.0	'Bingo'	fresh	105	104	6	0.99	0.94	1.00	0.06	0.02	0.13	0.06	0.02	0.13

Table 2. Elongation of shoots obtained from endosperm-derived callus in *Actinidia arguta* 'Bingo', 'Geneva', and 'Scarlet September Kiwi'.

Cultivar	Medium	No. of calli clumps with shoot buds	No. of elongated shoots
'Bingo'	SDM0	15	0
	SDM1	50	0
	SDM2	20	0
	SDM3	20	0
	SDM4	16	42
	SDM5	17	24
'Scarlet Sept. Kiwi'	SDM5	9	15
'Geneva'	SDM5	3	0

Table 3. Root induction of micro-cuttings shoots in *Actinidia arguta* 'Bingo', and 'Scarlet September Kiwi'.

Cultivar	Medium	No. of shoots cultured	No. of shoots forming roots
'Bingo'	RIM0	6	6
	RIM1	6	6
	RIM2	6	6
	RIM3	6	6
'Scarlet Sept. Kiwi'	RIM0	3	3
	RIM1	4	4
	RIM2	4	4
	RIM3	4	4

Table 4. Nuclear DNA content of *Actinidia arguta* 'Bingo' established in seedlings grown *in vitro* (control) and plants regenerated from endosperm-derived callus.

Plant material		No. of plants	DNA-ploidy	DNA content (pg)	
				2C (SD)	1Cx
Seedling (control)		9	4x	3.151 (0.124)	0.788
Source of endosperm explant		1	4x	3.117	0.779
	Fresh seed	14	6x	4.497 (0.035)	0.750
		17	4x	3.163 (0.042)	0.791
	Dry seed	3	6x	4.527 (0.033)	0.754

Figures

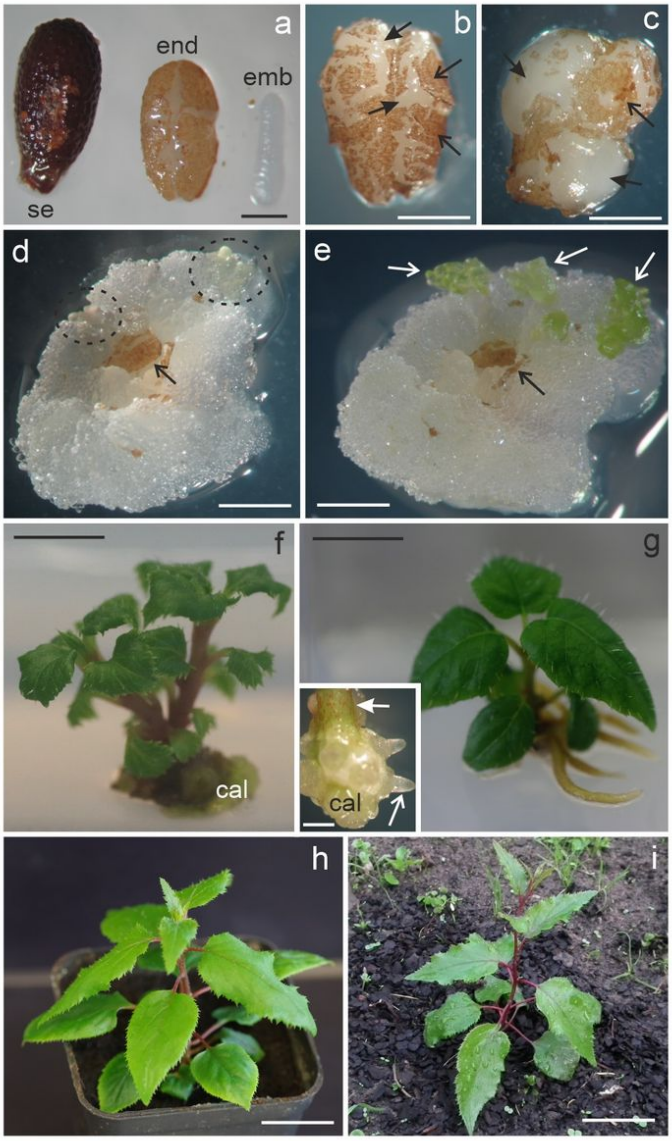

Figure 1

Callus induction and plant regeneration from cultured endosperm of Actinidia arguta 'Bingo' (a) whole seed (se), endosperm (end) after removing seed coat and dissecting embryo (emb). Callus proliferation (black arrows) after seven (b) and ten (c) days of culturing on CIM; visible remnants of seed coat (open black arrows). Callus with shoot primordia (dotted line) (d) and shoot buds (white open arrows) (e), after four and six weeks of culturing, respectively. (f) Shoots with callus (cal) on the basal end of the stem on SDM4. (g) Rooted plant on RIM1; magnification of a basal end of stem (white arrow) with callus and root induction (open white arrow) in inset. Plant acclimatized in a pot (h) and in soil in a garden (i). Scale bars = 200 µm (a); 500 µm (b, c); 1 mm (d, e); 1 cm (f, g); 1 mm (g inset); 2 cm (h, i)

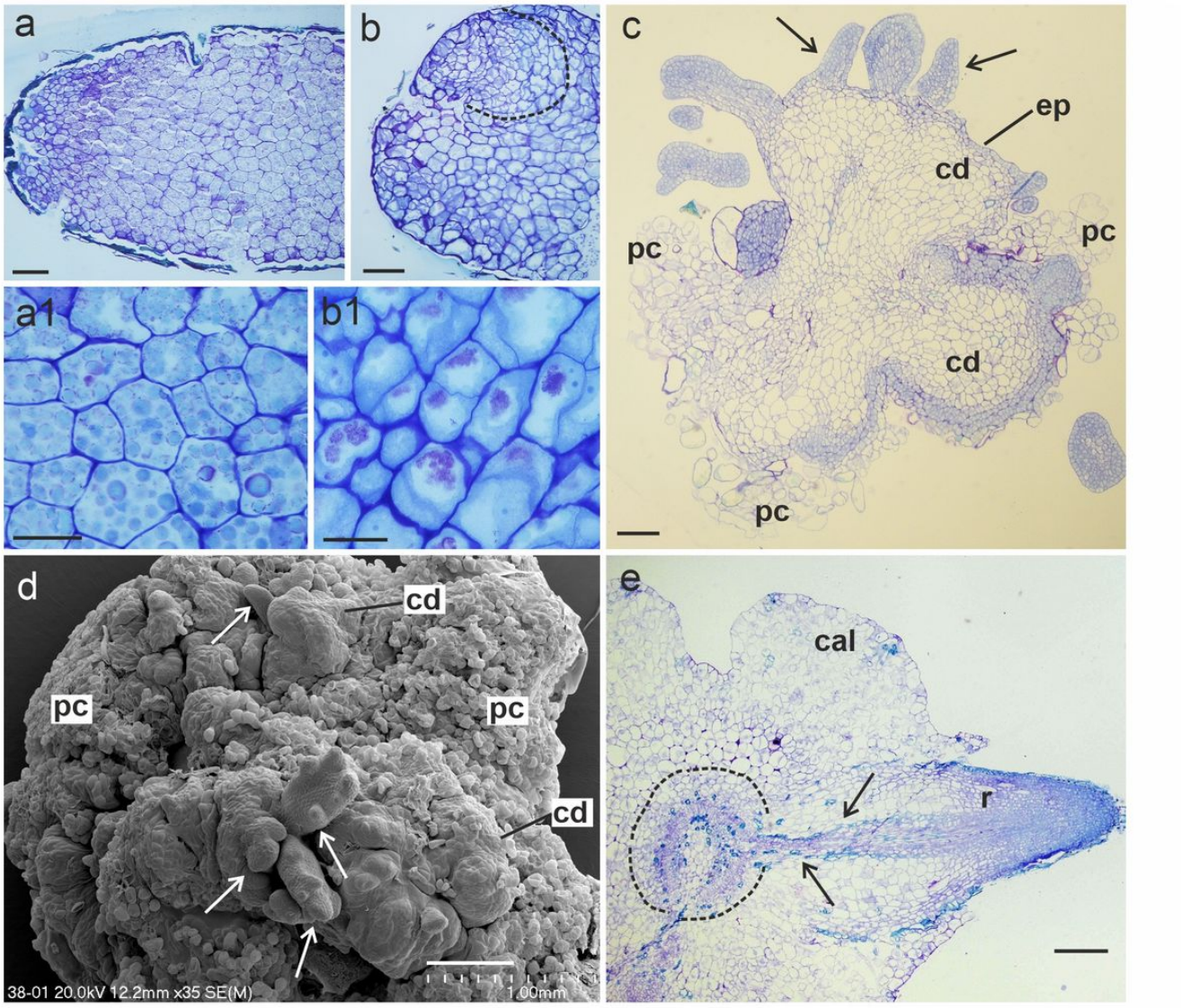

Figure 2

Histological (a–c, e) and ultrastructural (d) analyses of proliferation and regeneration from endosperm-derived callus in Actinidia arguta 'Bingo'. a Freshly isolated endosperm; magnification (a1) revealed cells abundant in storage materials. (b) Explant after 14 days of culturing on CIM-0.5; notice the induction of a callus (dashed line); magnification (b1) revealed vacuolated cells after division. (c, d) Adventitious shoot buds (arrows) arising from the callus domains (cd) covered with epidermis-like layer (ep) after six weeks culturing on CIM-0.5; parenchymatic cells (pc) are visible. (e) Histological section of the basal part of the stem with root induction on RIM1; notice that the vascular tissues of the root (r) are connected with the vascular tissue of the stem (dashed line) surrounded by the callus (cal). Scale bars = 50 μm (a1, b1); 100 μm (a, b); 200 μm (c, e); 500 μm (d)

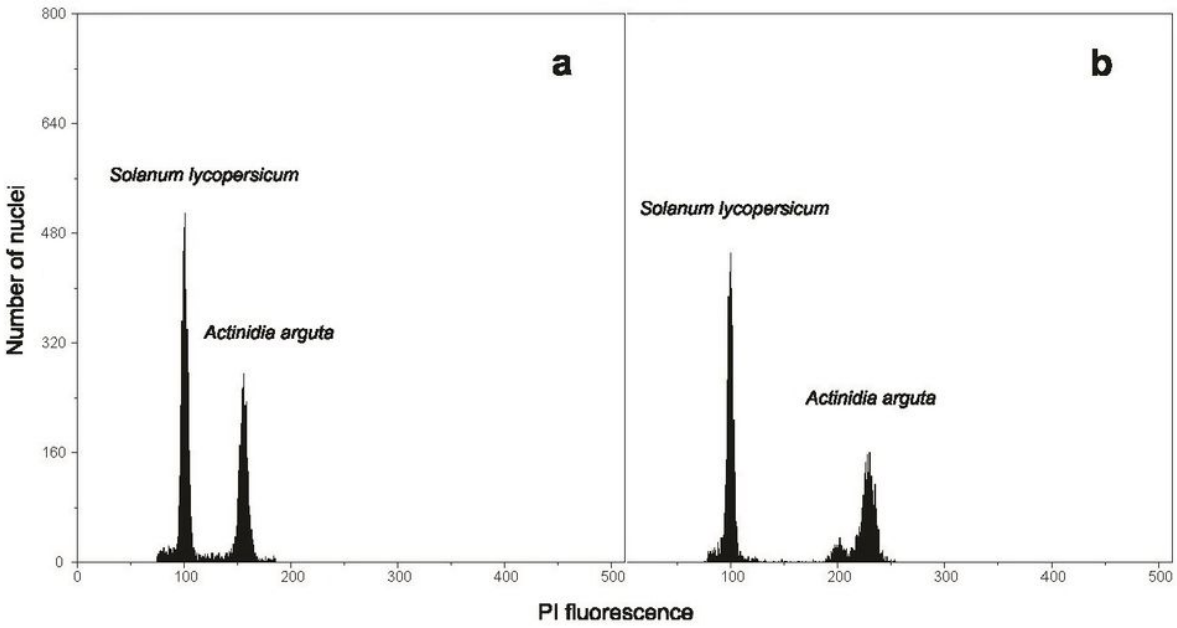

Figure 3

FCM histograms of nuclear DNA content obtained after analysis of PI-stained nuclei isolated from leaves of Solanum lycopersicum (internal standard) and (a) tetraploid seedling of Actinidia arguta 'Bingo' (2C=4Cx=3.1 pg), (b) hexaploid endosperm-derived Actinidia arguta regenerant (2C=6Cx=4.5 pg)