Creation of pepper doubled haploids and morphological characterization of androgenic plants

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Abstract The Balkan pepper breeding program aims to use in vitro embryogenesis (anther culture) to develop doubled haploid pepper lines with valuable traits. We analyzed the effectiveness of microspore embryogenesis on 17 pepper genotypes comprised of varieties, breeding lines, and F₁ hybrids of different varietal types (conical, bell shape, and kapia) and characterized the agronomic traits of newly generated doubled haploid lines. The highest androgenic potential was observed in the variety Stryama and breeding line 560/06 with 9.55 and 13.00% reacted anthers, respectively. Of the 186 regenerated plants, 147 were successfully adapted with a survival index of 79.03%. Flow cytometry analysis showed that the haploid:diploid ratio of regenerants was 1.5:1. All diploid plants were confirmed to be androgenic in origin. There were significant differences in terms of quantitative fruit characteristics of length, width, weight, pericarp weight, pericarp thickness, and productivity per plant among the diploid lines. Also, androgenic lines 21, 23, and 74 derived from Stryama and line 55 obtained from Zlaten medal 7 were distinguished with higher values of some fruit traits compared to initial genotypes. Additionally, several androgenesis lines (Stryama lines 21, 23, and 74, and Zlaten medal 7 line 55) scored higher for some fruit traits compared to the initial genotype. These results show that anther culture is a promising tool for the creation of Balkan pepper breeding lines with improved traits.

Keywords Androgenesis · Doubled haploid breeding · Haploid:diploid regenerants · Balkan pepper · Kapia

Abbreviations
DH Doubled haploid
PPFD Photosynthetic proton flux density
CV Coefficient of variation

Introduction

Conventional breeding has been pivotal for inbred line development and variety improvement. However, the time and resources required developing uniform and homozygous breeding lines is considerably longer and expensive (Cravero et al. 2011; Khan et al. 2017). New breeding techniques, such as marker-assisted selection (Lee et al. 2008) or molecular based biotechnological techniques such as mutagenesis (Riaz and Gul 2015)
and gene editing (Arora and Narula 2017) have emerged in the recent past and are complementing conventional breeding processes (Barroso et al. 2019; Öcal et al. 2019). Speed breeding and the adoption of speed breeding methods has become increasingly popular among practical breeding programs (Ahmar et al. 2020). Biotechnological techniques are economical to increase the breeding efficacy, but the initial investment required to maintain a biotechnological breeding pipeline is substantially higher. The combined use of biotechnological techniques and conventional breeding is becoming a new norm in plant breeding (Bermejo et al. 2016; Niazian and Shariatpanahi 2020).

Microspore embryogenesis is one of the most efficient and widely used methods to introduce genetic variability (Asif 2013; Dunwell 2010; Popova et al. 2016) and microspore-derived embryos and doubled haploid (DH) plants have proven useful in plant breeding. Microspore embryogenesis not only expedites the breeding process and achieve the homozygosity but also increases the breeding efficiency (Germanà 2011), which is important when creating a large number of genetically stable homozygous plants (Mityko and Gemesne Juhasz 2006). Several factors influence the successful induction of androgenesis and subsequent regeneration of plants; however, genotype, microspore development stages, and culture conditions seem to have a significant impact (Irikova et al. 2011a; Segui-Simmaro et al. 2011; Taskin et al. 2011) and the effect of these factors has been extensively studied in several crops.

Compared to other Solanaceae species, pepper (Capsicum annuum L.) is highly heterozygous and displays higher rates of spontaneous chromosome duplication among regenerants (Vagera 1990). According to Dolcet-Sanjuan et al. (1997), only one of 253 diploid plants was regenerated from somatic tissue or diploid pollen. The rates of spontaneous genome diploidization in regenerants ranges from 35 to 65% depending on the genotype analyzed (Irikova et al. 2011b). Confirmation of the microspore origin of diploid androgenic lines can be achieved through analysis of plant phenotypic homogeneity and fruit morphological characterization in the R2 generation and is particularly noticeable in the hybrid donor genotype (Supena et al. 2006a; Kisiala et al. 2011). Isozyme and molecular analysis has also been used to test the homozygosity (Dolcet-Sanjuan et al. 1997; Gemesne Juhasz et al., 2011; Parra-Vega et al. 2013a; Keleș et al. 2015).

Although recent advances in in vitro techniques have demonstrated their role in shortening the breeding cycles unequivocally (Barroso et al. 2019; Niazian and Shariatpanahi 2020); however, these techniques are still largely underutilized in most pepper breeding programs, which are still reliant on conventional breeding (Sánchez et al. 2020). This is particularly prevalent in Bulgaria and across the rest of the Balkans (Nankar et al. 2020). The aim of this study was to investigate the efficacy of anther culture techniques on Balkan pepper varieties for development of improved breeding lines. We analyzed the androgenic efficiency of 17 pepper genotypes comprising eight pepper varieties, one breeding line, and eight F1 hybrids, and characterized the newly generated doubled haploid breeding lines for their agronomic potential.

Materials and methods

Plant material

All experimental work was carried out during 2010–2014. A total of 17 pepper genotypes were used as anther donor plants: varieties Stryama, Hebar, Viktoria, Kurtovska kapia 1619, Kurtovska kapia 1, Kapia 1300, Kapia UV, and Zlaten medal 7; breeding line 560/06, and F1 hybrids 1647×Stryama, 1647×Hebar, 1647×Viktoria, 1647×Kurtovska kapia 1619, 1647×Kurtovska kapia 1, 1647×Kapia 1300, 1647×Kapia UV, and 1647×560/06. Donor plants were grown in 5-l pots in a mixture of peat moss and perlite in the ratio of 1:1 (v/v) in the greenhouse under natural light conditions and the average temperature of 22.5 °C was reported with a temperature ranging 15 to 30 °C during the period of April to October in respective years.

Anther culture

Anthers with microspores at uninuclear and early binuclear stages from flower buds of 3.5–4.0 mm length (Rodeva et al. 2007) were excised. The excised anthers were surface sterilized with 5% calcium hypochlorite for 20 min and rinsed three times in sterile distilled water for 5, 10, and 15 min. A similar sample collection protocol was implemented during
the 3-year period of anther cultivation (2010–2012). The microspore embryogenesis induction medium (MS0) contained micro- and macro salts and vitamins as per the recommendation of Murashige and Skoog (1962) and Gamborg et al. (1968), respectively. A composition of 0.1 mg l\(^{-1}\) 2,4-dichlorphenoxyacetic acid, 0.1 mg l\(^{-1}\) kinetin, 0.005 mg l\(^{-1}\) biotin, 0.1 mg l\(^{-1}\) glycine, 0.04 mg l\(^{-1}\) vitamin B12, 30 g l\(^{-1}\) sucrose, and 0.7% agar was used (Duchefa Biochemie, Plovdiv, Bulgaria) (Dumas De Vaulx et al. 1981). For each genotype, anthers from four different flower buds (20 explants) were placed in a Petri dish (6 cm in diameter) with the concave face down touching the culture medium. Depending on the genotype, 400 to 3337 anthers were cultivated. The anthers were treated in darkness at 35 ± 1°C for 8 days, followed by acclimatization at 26 ± 1°C and 18 ± 1°C during day and night, respectively. A photosynthetic proton flux density (PPFD) of 200 μmol m\(^{-2}\) s\(^{-1}\) and 14/10 h photoperiod was used. After 12 days, the anthers were incubated on the same medium without growth regulators (Dumas De Vaulx et al. 1981). Regenerants were transferred to 250-ml glass vessels containing 25 ml of rooting media including ½ MS0 medium, 20 g l\(^{-1}\) sucrose, 0.7% agar, and incubated in a growth chamber when they had reached 2 cm in length. All culture media was adjusted to pH 5.8 prior to autoclaving at 121 °C for 20 min. The proportion of reacted anthers (to the in vitro cultivated anthers), frequency of direct embryogenesis (embryos/100 anthers), and frequency of obtained plant regenerants (regenerated plants/100 anthers) were calculated as a percentage over the course of the multi-year experiment.

Plant adaptation and acclimatization

In vitro androgenic regenerants of 5–6 cm and seedlings developed from in vitro seeds (controls, six plants for each genotype) were extracted from the culture medium. Seedling roots were thoroughly rinsed with running water to eliminate residue of the culture medium. Plantlets were transferred to 0.5-l adaptation pots composed of a 1:3 (v/v) peat moss and perlite mixture and were covered with plastic film (Sigma Aldrich, Sofia, Bulgaria) for 10–12 days. Thereafter, plantlets and seedlings were transplanted to 5-l plastic pots composed of a 1:1 (v/v) peat moss and perlite mixture and were grown for 3 months (February to April) under control conditions at a temperature of 23 ± 1°C, a photosynthetic proton flux density (PPFD) of 200 μmol m\(^{-2}\) s\(^{-1}\), and 14/10 h photoperiod. In the beginning of May, adapted plants (R\(_1\) progeny) were transferred to the greenhouse and the survival index (% of survived plants) was determined after 2 weeks of adaptation.

Ploidy level analysis

The ploidy level of the obtained plant-regenerants was determined by flow cytometry (Partec PA-2, Görlitz, Germany). Flow cytometry analysis was performed on newly grown chopped leaves with 1 ml of nuclei extraction buffer (Solution A) filtered through a 50-μm Cell Trics filter (Partec, Germany), and mixed with DAPI staining buffer (Solution B). Traits of plant height (cm), length (cm), and width of leaf blade (cm) were also measured from the R\(_1\) regenerants.

Agronomic characterization of R\(_2\) regenerants

Plants of the original genotypes and 40 DH lines obtained from diploid regenerants of R\(_1\) progeny were evaluated for the agronomic traits. The material included a total of 40 lines comprising 20, six, and one lines originated from Stryama, Zlaten medal 7, and Viktoria varieties, respectively. Also, seven lines were derived from breeding line 560/06 as well as two, three, and one lines were obtained from F\(_1\) hybrids of 1647 × Viktoria, 1647 × Kapia UV, and 1647 × Kurtovska kapia 1619, respectively. Plant regenerants of each line and controls (from seeds) were grown and evaluated in an open field with two replications in randomized complete block design. Each replication consisted of 15 plants and was planted on the furrow surface in the scheme of 70/15 cm during 2014. Fruits were harvested before maturity and plant productivity (g), average fruit weight (g), fruit length (cm), fruit width (cm), usable part of the fruit (g), and fruit wall thickness (mm) was determined from five plants per replication and three fruits per plants.

Statistical analysis

Mean separation of fruit morphological and productivity was estimated using Duncan’s multiple range test and coefficient of variation (CV, %) was
determined using SPSS. Histograms of fruit traits (conical, bell shape, and kapia types) were generated using XLSTAT version 15.

Results

Anther culture

All studied pepper varieties, breeding lines, and F1 hybrids reacted to the applied conditions imposed for direct embryogenesis (Table 1, Fig. 1a, b). Anther response ranged from 0.17% (variety Hebar) to 13.00% (line 560/06). The highest percentage of reacted anthers and plant regenerants was obtained in variety Stryama and line 560/06, which showed fair androgenic potential (Tables 1 and 2), whereas varieties Hebar and Kapia UV were considered as non-androgenic [according to Mityko´ et al. (1995); scale of 5% plant regenerants]. The number of regenerant embryos into plants obtained from each genotype ranged from 1.72% (Kurtovska kapia 1) to 24.0% (Kurtovska kapia 1619).

Direct embryogenesis was observed in 447 out of 26,823 cultivated anthers (1.67%) (Table 1), resulting in 2119 formed embryos (7.90%) (Table 1). Plants developed from 186 (8.78%) embryos, of which 147 plants adapted and acclimatized successfully (survival index 79%) (Table 2 and Fig. 1c). Genotypes with conical pepper type had the highest proportion of reacted anthers, embryos formed, and percentage of successful plant regenerants with 4.92, 25.69, and 2.34%, respectively. Beside conical type, genotypes from kapia type also had a large number of reacted anthers; however, differences were non-significant and genotypes from kapia type were with the least androgenic reaction (Table 1). Overall, varieties included in this study showed better androgenic

| Genotype       | Cultivated anthers | Reacted anthers | Formed embryos |
|----------------|--------------------|-----------------|----------------|
|                | No                 | No %            | No Embryos/100 anthers |
| **Conical type** |                    |                 |                         |
| Stryama        | 1800               | 172 9.55        | 979 54.39              |
| F1 1647 × Stryama | 788               | 19 2.41         | 72 9.14                |
| Hebar          | 594                | 1 0.17          | 1 0.17                 |
| F1 1647 × Hebar | 491                | 10 2.04         | 19 3.87                |
| Zlaten medal 7 | 777                | 17 2.19         | 72 9.27                |
|                | 4449               | 219 4.92        | 1143 25.69             |
| **Bell shape type** |                |                 |                         |
| Viktoria       | 528                | 12 2.27         | 15 2.84                |
| F1 1647 × Viktoria | 517            | 10 1.93         | 63 12.19               |
| 560/06         | 400                | 52 13.00        | 362 90.50              |
| F1 1647 × 560/06 | 520               | 14 2.69         | 37 7.12                |
|                | 1965               | 88 4.48         | 477 24.27              |
| **Kapia type**  |                    |                 |                         |
| Kapia 1300     | 2466               | 22 0.89         | 106 4.30               |
| F1 1647 × Kapia 1300 | 2962          | 24 0.81         | 43 1.45                |
| Kurtovska kapia 1619 | 3337           | 18 0.54         | 25 0.75                |
| F1 1647 × K. kapia 1619 | 2517       | 25 0.99         | 96 3.81                |
| Kapia UV       | 2011               | 18 0.90         | 80 3.98                |
| F1 1647 × Kapia UV | 3030           | 12 0.40         | 77 2.54                |
| Kurtovska kapia 1 | 2123             | 13 0.61         | 58 2.73                |
| F1 1647 × K. kapia 1 | 1962           | 8 0.41          | 14 0.71                |
|                | 20,408             | 140 0.69        | 499 2.45               |
| **Total**      | 26,823             | 447 1.67        | 2119 7.90              |
Fig. 1 Stepwise protocol of pepper in vitro anther culture androgenesis to produce DH pepper regenerants. 

- a Direct embryo formation
- b embryo conversion
- c adapted plants
- d flow cytometric analysis of haploid regenerants
- e haploid regenerants
- f diploid regenerants
potential in comparison to F$_1$ hybrids, except for hybrid combination of 1647 × Hebar, 1647 × Vik-
toria, and 1647 × K. kapia 1619. Genotypes produc-
ing conical and bell-shaped peppers had similar
proportions for anther reactivity, embryos formed,
and successful regenerants. In contrast, genotypes of
the kapia type had much lower androgenic potential
(Table 1).

Analysis of chromosome level in regenerants

Ploidy levels of 147 acclimatized regenerants was
analyzed by flow cytometry. Of these plants, 88 were
haploid (59.86%) and 59 were as diploid (40.14%)
(Table 2 and Fig. 1d). The ratio of haploid:diploid
plants among varieties and F$_1$ hybrids was reported of
1.3:1 and 2.5:1, respectively. The occurrence of
spontaneous diploids was higher than haploids in
Zlaten medal 7, Viktoria, and Kurtovska kapia 1
(Table 2). Depending on the variety type, the ratio of
haploid:diploid plants was 1.4:1, 1.2:1, and 2.1:1 in
conical, bell shape, and kapia type genotypes,
respectively.

Agronomic characterization of R1 regenerants

The evaluation of morphological traits among haploid,
spontaneous diploids (R$_1$ regenerants), and controls is
presented in Table 3. Haploid plants from initial genotypes were characterized by a slight increase in height (ranging from 1.0 to 41.7 cm) whereas narrow leaves and pollen fertility (varied from 0.0 to 32.0%) (Fig. 1c). A detailed agronomic characterization of diploid plants displayed pollen fertility varying from 41.9 to 91.9%, but smaller leaf blade and low plant height phenotypes were commonly seen among DH

| Genotype                  | Plants | Ploidy level | Plant height (cm) | Leaf blade (cm) | Pollen fertility (%) |
|---------------------------|--------|--------------|-------------------|-----------------|----------------------|
|                           |        |              |                   | Length | Width |
| Stryama 6                 | Control| 40.0         | 17.0              | 7.3    | 93.0   |
| Regenerants               | 51     | n            | 18.3              | 6.9    | 3.4    | 10.6 |
|                           |        | 2n           | 18.6              | 8.6    | 4.7    | 77.1 |
| F₁ 1647 × Stryama         | 6      | Control      | 40.0              | 11.7   | 7.7    | 85.0 |
| Regenerant                | 1      | n            | 25.0              | 3.8    | 2.0    | 2.5  |
| F₁ 1647 × Hebar           | 6      | Control      | 39.0              | 14.7   | 8.0    | 82.4 |
| Regenerant                | 1      | n            | 1.0               | 4.0    | 2.8    | 0.0  |
| Zlaten medal 7            | 6      | Control      | 65.0              | 9.0    | 6.7    | 82.9 |
| Regenerants               | 2      | n            | 7.5               | 5.6    | 3.4    | 8.9  |
|                           |        | 2n           | 27.3              | 7.9    | 4.5    | 61.8 |
| Viktoria                  | 6      | Control      | 84.0              | 11.7   | 7.0    | 79.3 |
| Regenerant                | 1      | 2n           | 25.0              | 8.3    | 4.8    | 75.6 |
| F₁ 1647 × Viktoria        | 6      | Control      | 85.0              | 19.7   | 8.0    | 81.7 |
| Regenerants               | 2      | n            | 5.0               | 3.4    | 2.3    | 1.7  |
|                           |        | 2n           | 14.0              | 6.7    | 4.6    | 83.7 |
| 560/06                    | 6      | Control      | 74.0              | 10.0   | 5.8    | 80.0 |
| Regenerants               | 8      | n            | 8.8               | 5.5    | 3.2    | 12.5 |
|                           |        | 2n           | 13.3              | 8.2    | 5.2    | 82.7 |
| F₁ 1647 × 560/06          | 6      | Control      | 72.0              | 10.3   | 5.2    | 78.3 |
| Regenerants               | 2      | n            | 3.8               | 3.4    | 2.3    | 1.7  |
| Kapia 1300                | 6      | Control      | 34.0              | 15.0   | 5.7    | 84.7 |
| Regenerants               | 4      | n            | 21.3              | 9.8    | 3.4    | 32.0 |
|                           | 2      | 2n           | 25.0              | 8.9    | 3.3    | 91.9 |
| K. kapia 1619             | 6      | Control      | 40.0              | 20.3   | 6.5    | 87.3 |
| Regenerants               | 3      | n            | 33.7              | 8.0    | 2.3    | 8.1  |
|                           | 1      | 2n           | 58.0              | 13.0   | 5.3    | 69.6 |
| F₁1647 × K. kapia1619     | 6      | Control      | 34.0              | 20.0   | 7.8    | 93.7 |
| Regenerants               | 7      | n            | 28.6              | 9.0    | 4.3    | 6.1  |
|                           | 1      | 2n           | 13.0              | 8.7    | 5.2    | 41.9 |
| 1647 × Kapia UV           | 6      | Control      | 45.0              | 20.0   | 7.0    | 88.9 |
| Regenerants               | 6      | n            | 41.7              | 10.8   | 3.9    | 26.7 |
|                           | 4      | 2n           | 57.5              | 13.3   | 5.1    | 89.3 |
| K. kapia 1                | 6      | Control      | 40.0              | 21.3   | 7.2    | 91.3 |
| Regenerant                | 1      | 2n           | 35.0              | 9.3    | 3.7    | 88.5 |
| F₁ 1647 × K. kapia 1      | 6      | Control      | 33.0              | 21.3   | 6.7    | 91.2 |
| Regenerants               | 1      | n            | 32.0              | 9.0    | 3.3    | 10.0 |
|                           | 1      | 2n           | 30.0              | 11.7   | 5.3    | 84.6 |
plants (Fig. 2a2–b2) in comparison to controls (Fig. 1f and Fig. 2a1–b1). Only diploid regenerants from Kurtovska kapia 1619 and F1 hybrid of 1647 x Kapia UV were characterized by taller plants with 58.0 and 57.5 cm plant height, respectively, as compared to the control plants (Table 3). Although no noticeable differences were seen among the fruits of original genotypes (Fig. 2c1–d1), and DH genotypes (Fig. 2c2–d2); however, the fruits of DH genotypes were larger and bigger than the haploids (Fig. 2c3–d3).

Forty R2 generation plants, belonging to seven parental and control genotypes, were produced by selfing 59 DH R1 plants. Plant productivity and fruit morphological characters of the R2 plants were evaluated in conical, bell shape, and kapia type genotypes (Tables 4, 5, and 6, respectively) and average values of morphological traits are given in Fig. 3a–f. The coefficient of variation (CV) for most traits was less than 20%, suggesting significant phenotypic homogeneity among the androgenic lines. Among all assessed traits in conical, bell shape, and kapia type, the coefficient of variation (CV) was less than 20% for fruit length and width (Tables 4, 5, and 6) except for line 25 (CV = 23.38%) of conical variety.

Fig. 2  Representative comparison between leaf and flower (a1, a2, and a3), whole plant (b1, b2, and b3), whole fruit (c1, c2, and c3), and longitudinal and latitudinal fruit section (d1 and d2) of original Stryama genotype (top row: a1–d1), DH genotype (middle row: a2–d2), and haploid genotype (bottom row: a3–c3)
Higher variability (>30%) among the studied traits was recorded for productivity per plant (nine lines), fruit weight (one line), and usable part of the fruit, and fruit wall thickness (two lines), respectively. Duncan’s multiple range test revealed appreciable phenotypic diversity for fruit characters and productivity among androgenic lines, which are of the same origin.

Based on the morphological analyses of the studied androgenic lines, line 21 derived from conical shape was distinguished with the highest fruit weight of 110.3 g (Table 4) and overall lines belonging to conical shape discerned highest fruit weight (Fig. 3a) whereas lines derived from kapia type weighed the least. In comparison to original genotypes Stryama and Zlaten medal 7, higher fruit weight was observed.
among 19 lines and nine of them discerned significant differences. Conical type lines had longer fruits (Fig. 3b) with line 51 having the longest fruit (13.86 cm length) while fruit width was wider among bell shape lines (Fig. 3c) although line 49 of conical type formed the most wider fruits (5.71 cm). Only fruits from lines 49, 50, and 74 showed significantly wider fruits in comparison to original varieties of conical type. In comparison to the control, 12 lines (60%) reported higher weight of usable part of the fruit while considerable variation was observed for the fruit wall thickness with a wide range from 3.10 mm (line 560/06) to 13.88 mm (Viktoria 1647 × Viktoria 35).

**Table 5** Descriptive statistics of morphological and agronomic traits measured from bell-shape androgenic regenerants of R2 generation lines

| Line | Fruit Productivity/plant | Weight | Length | Width |
|------|--------------------------|--------|--------|-------|
|      |                          | g      | cm     | cm    |
|      |                          | CV     | CV     | CV    |
| 26   |                          | 105.6ab| 3.84   | 11.60ns| 5.52  | 6.00b  | 4.83  | 74.3a  | 2.19  | 4.20ns | 23.81  | 1383b  | 31.86  |
| 27   |                          | 90.0b  | 37.56  | 10.87ns| 16.93  | 5.77b  | 13.52  | 66.3b  | 38.79  | 4.30ns | 34.65  | 1690b  | 19.02  |
| 28   |                          | 87.2b  | 20.13  | 10.79ns| 3.06   | 5.51b  | 15.97  | 64.8b  | 14.54  | 5.00ns | 14.00  | 1240c  | 32.29  |
| 29   |                          | 99.9b  | 5.47   | 11.40ns| 9.91   | 6.38b  | 3.45   | 68.2b  | 21.34  | 4.67ns | 7.49   | 1466b  | 8.58   |
| 30   |                          | 90.7b  | 11.50  | 11.33ns| 5.12   | 5.59b  | 6.44   | 70.9b  | 12.26  | 4.23ns | 11.82  | 1640b  | 1.06   |
| 31   |                          | 88.8b  | 17.71  | 11.30ns| 8.85   | 5.65b  | 9.38   | 64.7b  | 15.01  | 3.67ns | 17.71  | 1466b  | 37.55  |
| 32   |                          | 101.4ab| 26.89  | 10.92ns| 3.57   | 5.88b  | 12.24  | 71.0b  | 15.26  | 3.87ns | 13.18  | 1480b  | 25.16  |
| 560/06 |                      | 131.9a | 0.28   | 11.67ns| 16.11  | 7.33a  | 0.82   | 96.1a  | 3.25   | 4.67ns | 12.42  | 2333a  | 6.55   |
| 33   |                          | 50.20b | 9.81   | 10.63ns| 7.14   | 4.23b  | 6.52   | 37.74b | 8.83   | 4.18ns | 18.91  | 1350b  | 9.80   |
| 34   |                          | 67.34a | 11.59  | 10.38ns| 13.51  | 5.15a  | 11.00  | 48.50a | 13.27  | 4.08ns | 14.18  | 1216b  | 12.55  |
| 1647 × Viktoria | | 45.61b | 7.77   | 11.76ns| 5.93   | 3.93b  | 4.81   | 36.37b | 11.73  | 4.00ns | 20.00  | 2106a  | 4.78   |
| 36   |                          | 66.67b | 19.13  | 10.19ns| 16.05  | 5.01b  | 3.16   | 50.79b | 24.56  | 4.50ns | 3.67   | 1350b  | 8.73   |
| Viktoria |                      | 85.15a | 3.41   | 10.62ns| 2.50   | 6.01a  | 1.92   | 63.67a | 2.29   | 4.50ns | 13.88  | 1950a  | 3.69   |

a,b,cValues in columns followed by different letter are significantly different at *P < 0.05, Duncan’s multiple range test

ns not significant

**Table 6** Descriptive statistics of morphological and agronomic traits measured from kapia type androgenic regenerants of R2 generation lines

| Line        | Fruit Productivity/plant | Weight | Length | Width |
|-------------|--------------------------|--------|--------|-------|
|             |                          | g      | cm     | cm    |
|             |                          | CV     | CV     | CV    |
| 76          |                          | 51.2ns | 11.68  | 12.30ab| 10.94 | 4.43ns | 11.35 | 39.3a  | 7.75  | 3.00    | 16.67 | 188b  | 10.11 |
| 77          |                          | 51.9ns | 2.75   | 10.30ab| 18.54 | 4.77ns | 15.00 | 37.0ab | 5.80  | 3.20    | 24.12 | 404ab | 26.26 |
| 78          |                          | 49.7ns | 18.02  | 12.80a | 5.47  | 4.53ns | 6.37  | 37.4ab | 17.74 | 2.66    | 21.68 | 348ab | 11.19 |
| 1647 × Kapia UV |              | 46.1ns | 7.04   | 10.73ab| 5.69  | 4.50ns | 5.88  | 28.5b  | 12.15 | 2.33    | 7.72  | 508b  | 11.32 |
| 35          |                          | 36.6b  | 11.58  | 10.70ab| 6.47  | 3.87b  | 4.20  | 28.92b | 11.89 | 3.33ns  | 0.00  | 1180b | 15.56 |
| 1647 × K. kapia 1619 |         | 49.17a | 1.427  | 12.63a | 1.78  | 4.31a  | 1.98  | 36.31a | 4.29  | 3.42ns  | 3.99  | 1350a  | 5.330 |

a,b,cValues in columns followed by different letter are significantly different at *P < 0.05, Duncan’s multiple range test

ns not significant
20) to 5.61 mm (line 21). Higher wall thickness was distinguished in lines 21, 46, and 50; however, it was the same among conical and bell shape types (Fig. 3d). The highest weight of the usable part of the fruit was observed in conical type (Fig. 3e) with line 23 (87.8 g) having highest weight and line 20 the least (30.0 g). Higher productivity was registered in conical type lines 21, 22, 23, 24, and 25 with a range of 1375 to 2225 g; however, line 25 displayed the significant differences only as compared to the control (variety Stryama). Average productivity was reported highest in the bell shape (Fig. 3f) and it was followed by the conical type. For breeding purposes, noticeable traits of interest were reported among lines 21, 23, and 74. Lines originated from variety Zlaten medal 7 did not perform better than the control for the studied traits except line 55, which discerned higher values for fruit weight, pericarp weight, and productivity; however, no significant differences were reported for any of the trait (Table 4).

In bell-shape varietal type, only androgenic line 34 displayed higher fruit weight, fruit width, and pericarp weight compared to the initial hybrid genotype of 1647 × Viktoria, but did not show higher productivity (Table 5). Other lines were also seen to characterize with low productivity compared to the control. Plants

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**Fig. 3** Histograms depicting the average values of morphological traits for genotypes of conical, bell shape, and kapia type.
from lines 36, 76, 77, and 78 were with higher values of some fruit parameters than the control 1647 × Kapia UV hybrid while the same lines showed low productivity. However, it must be noted that significant differences were observed for pericarp weight and thickness in lines 76, and 77 and low productivity for line 76 (Table 6). Plants from line 35 had lower values for the studied traits than the initial hybrid of 1647 × Kurtovska kapia 1619.

Discussion

This study confirms the effectiveness of microspore embryogenesis for the generation of haploid/DH embryos and homozygous regenerants in pepper through another culture; however, many factors were observed that influenced the effectiveness of anther culture. These findings are in alignment with previous findings that reported the effect of donor plants (Cheng et al. 2013), microspore developmental stage (Parra-Vega et al. 2013a), culture medium (Irikova et al. 2011a; Nowaczyk et al. 2016; Ata et al. 2019; Grozeva and Nankar 2020), growing conditions and age (Irikova et al. 2011b; Parra-Vega et al. 2013b; Popova et al. 2016; Grozeva and Nankar 2020). Niklas-Nowak et al. (2012) observed that the significant differences were seen in the androgenic response of tested genotypes of individual plants and confirmed that the donor plant genotype is a critical factor in determining the effectiveness of androgenesis in pepper, which is in coherence with our findings.

Data obtained from the investigation of 17 pepper genotypes discerned significant differences in androgenic response. Anther reactivity for each line varied from 0.17% (Hebar) to 13% (560/06). In total, we obtained 186 regenerants from 2119 formed embryos (8.78%). The highest yield of normal looking embryos per flower bud was reported of 0.9 in breeding line 560/06, and similar observation was made by Supena et al. (2006b), and Ari et al. (2016) studies of pepper shed-microspore culture. Olszewska et al. (2014) studied the androgenic efficiency of 17 Capsicum genotypes and reported androgenic response varying from 0 to 6.15% and was able to develop 46 regenerated plants (40.0%) from 115 viable embryos, which is significantly higher than the obtained 8.78% regenerants frequency of our study. Erçan and Ayar Sensoy (2011) reported similar results with androgenic response varying from 0 to 7.69% and noted the effect of genotype-specific background; however, the numbers of regenerants were very few. Mityko and Fari’s (1997) comprehensively studied the anther cultivation of 500 varieties, breeding lines, and F1 hybrids and reported that androgenic response was reduced from 76 to 0 plants per 100 anthers in the order of wax type > dark green type > tomato shape > hot pepper. Our results indicated that the embryogenic efficiency decreased from conical > bell shape > kapia type, which is in line with Koleva-Gudeva et al. (2013). According to Mitykó et al. (1995), F1 hybrids produced between a cross of poor/non-responsive and responsive genotypes showed fair response despite poor response of donor parent. Results of the current research showed the similar trend; however, androgenic response of studied F1 hybrids was poor, which might have been due to the participation of genotype 1647 in hybrid combination since it possesses genetic male sterility, which may have influenced the number of formed embryos and plant regenerants.

The chromosome level of obtained androgenic regenerants was 59.9% haploids and 40.1% spontaneous DHs, which is consistent with Gyulai et al. (2000) reports of 64.5% haploids, 32.6% spontaneous DHs, 0.6% tetraploid, and 2.3% aneuploid plants. Chromosome level reported in our study showed slightly less haploids and more diploids than Gemesne Juhasz et al.’s (2001) study, which reported 68.5% haploid and 29.8% spontaneous DHs, 0.7% tetraploid, 1.0% aneuploid plants and a chimera. We did not report any tetraploids and aneuploids, which contradicts Gyulai et al.’s (2000) and Gemesne Juhasz et al.’s (2001) findings due to the fact that we did not use the colchicine for chromosome doubling. According to Mityko and Fari (1997), the haploid-to-DH regenerants ratio was genotype-dependent, and reported that the level of haploid to DHs varied from 1:1 to 1:2 in large fruit pepper and was 3:1 to 2:1 in small fruited pepper used for spices. Other sweet pepper F1 hybrid-based anther culture studies have also reported haploid regenerants ranging from 20 to 40% (Shrestha and Kang 2009; Shrestha et al. 2011). In addition, Luitel and Kang (2013a) revealed that the F1-derived sweet red-, yellow-, and orange-colored genotypes produced 44.5, 42.5, and 33.3% haploids and 55.5, 57.6, and 66.7% spontaneous DHs, respectively. Our results for studied F1 hybrids are in
accordance with the observed findings of Parra-Vega et al. (2013a); however, the differences in the regenerants ploidy level might not only be genotype-dependent, but also due to in vitro conditions of anther cultivation including culture medium, stress treatment, and other factors.

Haploid pepper plants are often morphologically differentiated by narrow leaves (Fig. 2a3), shorter internodes, reduced growth (Fig. 2b3), sterile pollens, and smaller fruits (Fig. 2c3). These observations are confirmed in the present work as well as in previous studies of Dolcet-Sanjuan et al. (1997) and Shrestha et al. (2011); however, presented results warrant further investigation of androgenic origin of diploid regenerants. Irikova et al. (2011b) has concluded that the ploidy level must draw attention and should be further investigated since in vitro cultivation stimulates the cell division of anther wall somatic tissues. Dolcet-Sanjuan et al. (1997) reported that only one out of 253 diploid plants were derived from diploid pollen or somatic tissues. According to Parra-Vega et al. (2013a), minimized or excluded callus formation in anther culture could reduce the induction of non-DHs and suggested this as an alternative method known as shed-microspore or microspore culture. A low percentage of normal-looking embryos in shed-microspore and subsequent plant regeneration of microspore-derived embryos is still a problem (Supena and Custers 2011; Ari et al. 2016). Evidently, Lantos et al. (2012) established around 20 to 100 embryo-like structures per Petri dish while only 0 to 8 green plants per Petri dish could be further developed.

Despite many advantages of anther culture, there are still many difficulties that are associated with doubling of haploid regenerants genome and a high level of plants with diploid chromosome set. Olszewska et al. (2010) and Supena et al. (2006a) used a high morphological homogeneity in R2 and subsequent generations method to confirm the microspore origin of diploid regenerants. Parra-Vega et al. (2013a) demonstrated a gametophytic origin of embryo-derived putative DH regenerants using SSR markers while Olszewska et al. (2017) used random amplified polymorphic DNA (RAPD) markers to prove androgenic origin of regenerants obtained from interspecific hybrid between C. annuum and C. frutescens. High phenotypic homogeneity in each R2 population was evidently visible in this study and it corresponds to the androgenic origin and DH nature of diploid plants, which is in agreement with Olszewska et al. (2011) and Kisiala et al. (2011). In most DH lines, significant variation was seen for productivity and it is due to the fact that yield and fruits per plant significantly depend on the environmental conditions and similar observation was made by Kisiala et al. (2011) and Grozeva et al. (2020) for the corresponding traits. F1 hybrid-derived androgenic lines in this study discerned slight variation and phenotypic uniformity for the measured traits, which proves that these are DH plants.

Morphological characterization of plants is a possibility not only to prove the spontaneous genome diploidization and achieving homozygosity, but also to evaluate the breeding potential of prospective lines. Observed results for agronomic and morphological traits showed noticeable variation for fruit morphological and productivity traits between diverse androgenic lines of similar background and these results are in accordance with Grozeva et al. (2020) and Nowaczek et al. (2016). Variation for fruit morphological and agronomical traits observed between genotypes of Stryama, Zlaten medal 7, and Victoria varieties background and similar observations have been observed in previous studies in regard to fruit morphological traits variation among androgenic lines of similar background (Shrestha et al. 2011; Luitel et al. 2012; Shmykova et al. 2014; Trajkova and Koleva-Gudeva 2017). The breeding value of different pepper androgenic material has been observed by many authors and promising breeding lines have been created for enhanced fruit quality (Shrestha et al. 2011; Luitel and Kang, 2013b; Nowaczek et al. 2014; Grozeva et al. 2020), improved productivity, plant and fruit traits (Koleva-Gudeva and Trajkova 2012; Luitel et al. 2012; Todorova et al. 2013; Shmykova et al. 2014; Trajkova and Koleva-Gudeva 2017; Grozeva et al. 2020). Applications of pepper androgenesis have not only been limited to fruit quality and fruit morphological traits, but have also been useful in creating DH lines that have resistance against economically important viral, bacterial (Hwang et al. 1998), and fungal diseases (Todorova et al. 2013), and nematodes (Ocal et al. 2019; Shimira et al. 2019).
Conclusions

In conclusion, our study has revealed that the frequency of embryo formation, plant regeneration, and ratio of haploid: diploid plants is genotype-specific and strongly depends on the genetic background. Morphological evaluation and trait characterization confirmed the androgenic origin of 40 plant regenerants in the R2 progeny and trait characterization allowed us to analyze the breeding potential of newly created DH lines. This research has been instrumental in creating a valuable germplasm, which could be a good foundation for further investigation of fundamental genetics research and utilize them in practical breeding application to expedite the breeding process and increase the genetic gain. Based on the outcome of this research, this study illustrates the usefulness of microspore embryogenesis and anther culture as a suitable method for creating initial breeding material and biodiversity enrichment for variety improvement and hybrid development.

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Author contributions

Study conception and design [Stanislava Grozeva]. Material preparation, performed of experiments, and data collection [Stanislava Grozeva and Velichka Todorova]. Data analysis and visualization [Amol N. Nankar]. Original manuscript draft written by [Stanislava Grozeva]. Reviewed manuscript drafts [Velichka Todorova and Amol N. Nankar]. All authors read and approved the final manuscript.

Data availability

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

Declaration

Conflict of interest

The authors declare that they have no conflicts of interest.

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