In Vitro Gynogenesis and Flow Cytometry Analysis of the Regenerated Haploids of Black Cumin (Nigella sativa)

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Abstract. In vitro ovule culture could be used to generate homozygous lines through the production of haploid plants. The present study reports on in vitro regeneration and production of haploid plants through ovule cultures and identification of the regenerated haploids using flow cytometry. The ovules were cultured on Murashige and Skoog (MS) medium supplemented with different concentrations of 6-benzyladenine (BA), kinetin (Kin), 2,4-dichlorophenoxyacetic acid (2,4-D), and naphthalene acetic acid (NAA) at 0, 0.5, 1, and 2 mg·L⁻¹ for their gynogenesis. Among different plant growth regulators (PGRs) tested, 2,4-D at 2 mg·L⁻¹ produced direct gynogenesis. The highest callogenesis percentage (100%) was obtained on MS medium containing 1 mg·L⁻¹ 2,4-D and 2 mg·L⁻¹ NAA. Flow cytometry analysis was used to identify the regenerated haploids. It also confirmed gynogenic occurrence at 1 and 2 mg·L⁻¹ 2,4-D with percentages of 21.7% and 41%, respectively. Therefore, 2,4-D proved effective for the induction of haploids in black cumin. The regenerated haploids were developed on MS medium without PGRs. The obtained results of in vitro gynogenesis and haploid plant production can tremendously facilitate breeding programs of black cumin.

Black cumin (Nigella sativa L.; 2n = 2x = 12) is an annual flowering plant belonging to the Ranunculaceae family. In Islamic religion, Prophet Muhammad once stated that the black seed can heal every disease, except death. The plant has been used traditionally as a spice, as a food preservative, and for treatment of various diseases for centuries in the Middle East, Northern Africa and India (Gilani et al., 2004). Several reports substantiated black cumin for its anticancer (Khan et al., 2011; Majdalawieh and Fayyad, 2016), immunomodulatory (Boskahady et al., 2011; Salem, 2005), and anti-inflammatory activity (Chehi et al., 2009; Pise and Padwal, 2017). Many species and mutants (Chloroxanthanum mutant) of black cumin can be grown as ornamental plants in gardens (El-Mahrouk et al., 2015; Subrahmanyam, 2009). Production of homozygous populations by inbreed method, for hybrid breeding programs, is time- and money consuming for all plants. Alternatively, true homozygous lines can be obtained by the resulting haploid plant. Haploid, a plant that has a basic chromosome number [gametic chromosome number of diploid plant (n)], represents a good method to accelerate plant breeding. Consequently, the importance of haploids is the production of pure lines [doubled-haploid (DH) plants] by chromosome diploidization of haploids, which have many useful advantages such as direct generation of new cultivars in self-pollinated crops, produce high-yielding hybrids among pure line crosses, or both (Veillieux, 1994). Haploids can be originated spontaneously in nature or as a result of various induction techniques. In vitro culture techniques for haploid production such as male gametophytes (androgenesis) and female gametophytes (gynogenesis) were reported. There are more than 200 species of angiosperms that produce haploid plants through in vitro androgenesis (Maluszyński et al., 2003). The first production of haploid plants through in vitro gynogenesis of unfertilized ovaries culture was reported in barley (Hordeum vulgare) (San Nocem, 1976). In vitro induction of haploid plants from unpolinated ovules has been applied to several other crops and considered to be an alternative to anther culture technique in cases where anther culture has not been successful (Yang and Zhou, 1982). Haploid plants can be induced by pathenogenic development of the egg cell or other female gametophyte cells which lead to haploid or DH plant production (Forster et al., 2007). Moreover, diploid plants may be resulted from somatic cell besides haploid or DH plants, which can be produced by spontaneous diploidization, so that identification of regenerants’ ploidy level is crucial. This can be done by various evaluation methods such as morphological traits of DH plants in the progeny (Murigneux et al., 1993; Rakha et al., 2012), isozymes (Kielkowska and Adamus, 2010), molecular markers using restricted fragment length polymorphism (Murigneux et al., 1993), simple sequence repeats (Muranty et al., 2002), and flow cytometry (Kielkowska and Adamus, 2010; Lotfi et al., 2003). The first use of DNA flow cytometry in plants was reported by Galbraith et al. (1983). The nuclei are classified according to their relative fluorescence intensity or DNA content (Dolezel and Bartos, 2005). DNA flow cytometry has become a popular method for ploidy screening, detection of mixoploidy, and aneuploid.

The previous reports studied the nutritional requirements and PGR effects for ovule formation in black cumin (Peterson, 1973, 1974). However, to our knowledge there are no reports on haploid induction in black cumin. Therefore, this study is the first report on haploid production of black cumin from in vitro ovule culture. The aim of this study was to develop an efficient protocol for
both in vitro gynogenesis embryo production and identification, using flow cytometry approaches.

**Materials and Methods**

**Plant material and production of female flower buds.** Pure line of diploid black cumin seeds resulting from three years of self-pollination were used as the plant material in this study (El-Mahrouk et al., 2015). Tetraploid seeds of black cumin were used in the comparative experiment by flow cytometry (Maamoun et al., 2014). Both diploid and tetraploid seeds were sown in the experimental farm of Kafrelsheikh University on the 15th of Oct. 2015 to obtain female flower buds. Donor plants were grown in open field conditions and all cultivation practices, i.e., plant density, irrigation, and fertilization were conducted according to Yimam et al. (2015).

**Ovule culture establishment.** Black cumin ovaries were picked one to 2 d before anthesis from 100 diploid plants and used directly or after exposing them to cold pretreatment at 4°C for 5 d according to Lux et al. (1990). The fresh and cold-treated flowers were prepared by removing petals and style. Then, the ovaries were surface-disinfected for 2 min in 70% ethanol, followed by 15 min in a solution of 70% (v/v) Clorox (sodium hypochlorite 0.05%, NaOCl) containing two to three drops of Tween-20 (Loba Chemical Company, Mumbai, Maharashtra, India). After three rinses with sterile distilled water, ovaries were dried on filter paper and the ovaries were excised in a laminar flow cabinet. The ovules were cultured in a 7-cm petri dish containing MS medium without PGRs for their subsequent growth. The cultures were kept at 25°C and 16-h photoperiod at 35 μmol·m⁻²·s⁻¹ photosynthetic photon flux density provided by fluorescent tubes. After 4 weeks of culture, survival percentage, plant height, number of leaves, and number of roots were recorded.

**Ploidy level determination.** The ploidy level of the regenerants was determined by an Attune flow cytometer (Applied Biosystem, CA). Samples were prepared from the young leaves according to the method of Galbraith et al. (1983). About 50 mg leaf tissue was chopped and macerated in lysis buffer (1 mL) to release intact nuclei by razor blade in Galbraith buffer [45 mM MgCl₂; 30 mM sodium citrate; 20 mM MOPS; 0.1% (w/v) Triton X-100; pH 7.0] for 1 h. The cell suspensions were filtered through a 0.45-μm nylon filter to eliminate cell debris for 5 min at room temperature. Then, the cell nuclei were stained with 10 μL 4',6'-diamino-2-phenylindole (DAPI) solution (solution A of high-resolution kit type P, Partec) for 30 min on ice in the dark. Leaves of diploid (2n = 2x = 12) plants of black cumin were used as a reference standard. Nearly 10,000 nuclei were analyzed using a logarithmic scale. Histograms were analyzed using Attune cytometric software version 2.1.

**Statistical analysis.** In vitro experiments were set up in a completely randomized design and repeated twice. Each treatment consisted of three replications and each replication was represented by three petri dishes rendering a group of 75 ovules per treatment. Analysis of variance was conducted using SPSS (version 20) statistical software.

### Table 1. Effect of plant growth regulators on callus induction and direct gynogenesis of black cumin ovules after 6 weeks.

| Treatment (mg·L⁻¹) | Callus formation (%) | Callus diam (mm) | Direct gynogenesis (%) |
|-------------------|----------------------|------------------|------------------------|
| Control           | 0.0                  | 0 c              | 0 c                    | 0 b                    |
| BA 0.5            | 0 c                  | 0 c              | 0 b                    |
| 1.0               | 0 c                  | 0 c              | 0 b                    |
| 2.0               | 0 c                  | 0 c              | 0 b                    |
| Kin 0.5           | 0 c                  | 0 c              | 0 b                    |
| 1.0               | 0 c                  | 0 c              | 0 b                    |
| 2.0               | 0 c                  | 0 c              | 0 b                    |
| 2.4-D 0.5         | 0 c                  | 0 c              | 0 b                    |
| 1.0               | 100 ± 0.00 a         | 4.7 ± 0.057 b    | 0 b                    |
| 2.0               | 66.6 ± 0.88 b        | 9.8 ± 0.176 a    | 33.4 ± 0.208 a         |
| NAA 0.5           | 0 c                  | 0 c              | 0 b                    |
| 1.0               | 0 c                  | 0 c              | 0 b                    |
| 2.0               | 100 ± 0.00 a         | 4.5 ± 0.29 b     | 0 b                    |
| Significant       | **                  | **              | **                    |
| LSD               | 0.743                | 0.028            | 0.175                  |

*Mean separation within each column by Fisher’s least significant difference (LSD) test at 5% level.

BA = benzyladenine; Kin = Kinetin; 2,4-D = 2,4-dichlorophenoxyacetic acid; NAA = naphthalene acetic acid.

**Gynogenesis and growth of haploid plants.** Responded ovules were transferred to petri dishes (10 explants/petri dish) containing MS medium supplemented with 5 mg·L⁻¹ NAA and 5 mg·L⁻¹ BA according to El-Mahrouk et al. (2010). The cultures were incubated under dark conditions. After 8 weeks of culture, the number of embryo per ovule, the number of embryo-like structures per callus, and the gynogenetic percentage were recorded. The regenerated embryos and embryo-like structures were transplanted into MS medium without PGRs for their subsequent growth. The cultures were kept at 25°C and 16-h photoperiod at 35 μmol·m⁻²·s⁻¹ photosynthetic photon flux density provided by fluorescent tubes. After 4 weeks of culture, survival percentage, plant height, number of leaves, and number of roots were recorded.

**Fig. 1. In vitro production of black cumin haploids; (A) in vitro ovule culture, (B) callus induction of ovules, (C) embryogenesis of callus responded on 2 mg·L⁻¹ 2,4-D after 8 weeks (red arrows refer to direct embryos), (D) embryogenesis of callus responded on 1 mg·L⁻¹ 2,4-D after 5 weeks, (E) elongation of embryos on Murashige and Skoog (MS) medium without plant growth regulator (PGR), and (F) elongation of embryo-like structures on MS medium without PGR (bar = 1 cm).**
program. The mean separations were carried out using Fisher’s least significant difference test and significance was determined at $P \leq 0.05$, (Fisher, 1954). Data were presented as means ± SE.

Results and Discussion

Effect of PGRs on callus induction and direct gynogenesis. The type of PGRs proved to be one of the most important factors influencing callus induction and direct embryogenesis in black cumin. The use of fresh flowers without cold pretreatment did not induce the ovule growth. Stress treatment is a common factor affecting gynogenesis in many species (Kwack and Fujieda, 1988; Lux et al., 1990). Ovule growth induction was achieved only by using auxins at 1 and $2 \cdot mg^{-1} L^{-1}$ 2,4-D and $2 mg^{-1} L^{-1}$ NAA treatments (Table 1). Calluses were developed on the ovule surface within 3 weeks of culture (Fig. 1B). The callus subsequently covered the whole surface of the ovule by the end of 6-week culture. The best result of callus formation (100%) was obtained on the medium supplemented with $1 mg^{-1} L^{-1}$ 2,4-D and $2 mg^{-1} L^{-1}$ NAA followed by $2 mg^{-1} L^{-1}$ 2,4-D, achieving 66.6% callus formation. On the other hand, no response was observed in all other treatments. The medium supplemented with $2 mg^{-1} L^{-1}$ 2,4-D induced the highest callus growth (9.8 mm callus diameter), whereas the treatments of $1 mg^{-1} L^{-1}$ 2,4-D and $2 mg^{-1} L^{-1}$ NAA developed 4.7 and 4.5 mm callus diameter, respectively. The embryogenic calluses were developed on the surfaces of the ovules 6 weeks after culture initiation. It was observed that gynogenesis of black cumin ovules can be obtained directly on medium containing $2 mg^{-1} L^{-1}$ 2,4-D achieving 33.4% (Table 1; Fig. 3C). Ovules of black cumin did not respond to cytokinin treatments and no growth could be achieved in the absence of auxins. This finding confirms previous results obtained by El-Mahrouk et al. (2010) on Arbutus undeo, that the highest percentage of callus formation was observed on the medium containing NAA or 2,4-D. The previous study mentioned that 2,4-D is important for promoting and stimulating the formation of callus tissues (Kiëlkowski and Adamus, 2010; Matsubara et al., 1995). Metwally et al. (1998) reported that the ovules producing embryogenic callus increases by increasing the auxin concentrations. In addition, 2,4-D has been proved effective for somatic embryogenesis in most plant species (Evans et al., 1981). According to Campion et al. (1992), 2,4-D gave the best result for haploid production of ovule, ovary, and whole flower bud of onion (Allium cepa). Haploid plants produced by gynogenesis can be regenerated directly or indirectly from callus (Keller and Korzun, 1996). All cells in the embryonic sac can be the main parts of direct gynogenesis that formed proembryos first and then differentiated to embryos (Akgo et al., 2017). On the other hand, callus can be formed directly from the egg cell, synergids, polar nuclei, and antipodals or can be developed from proembryos in indirect gynogenesis (Reed, 2005). Similar to our results, many studies mentioned that cold pretreatment of flower buds at 4 °C for 4–5 d led to increased embryo yield of sugar beet (Beta vulgaris) (Lux et al., 1990) and winter squash (Cucurbita moschata) (Kwack and Fujieda, 1988). However, in contrast to our results, ovules from flower buds without cold pretreatment showed a better embryogenic response in Cucurbita pepo (Gemes-Juhasz et al., 2002; Metwally et al., 1998). The results conclude that many factors can affect gynogenesis, including PGRs and cold pretreatment of flower buds and plant species.

Organogenesis of responded ovules. Under light conditions, all embryogenic calluses that formed on the surface of whole ovules developed into embryo-like structures (ELS) within 4 weeks of culture on organogenesis medium ($5 mg^{-1} L^{-1}$ NAA + $5 mg^{-1} L^{-1}$ BA) (Figs. 1C and D and 2). Numerous small clumps of compact cells were developed into a mass of ELS.
Table 2. Growth of the regenerated haploid embryos of black cumin on MS medium without PGRs.

| Treatment-derived embryos | Survival percentage (%) | Number of leaves/plantlet | Number of roots/plantlet | Plantlet ht (cm) |
|---------------------------|-------------------------|---------------------------|--------------------------|-----------------|
| 1 mg L^-1 2,4-D           | 84 ± 1.0 b              | 3.5 ± 0.057 b            | 1.2 ± 0.0145 b          | 3.3 ± 0.120 b   |
| 2 mg L^-1 2,4-D           | 89 ± 1.0 a              | 5.0 ± 0.088 a            | 2.4 ± 0.034 a           | 4.2 ± 0.1 a     |
| 2 mg L^-1 NAA             | 0 ± 0.00 c              | 0 ± 0.00 c               | 0 ± 0.00 c              | 0 ± 0.00 c      |

Significant ** ** ** **

LSD 1.19 0.088 0.030 0.11

^Mean separation within columns by Fisher’s least significant difference (LSD) test at 5% level.

MS = Murashige and Skoog; PGRs = plant growth regulators; 2,4-D = 2,4-dichlorophenoxyacetic acid; NAA = naphthalene acetic acid.

(5.93) was obtained from callus derived on MS medium supplemented with 2 mg L^-1 2,4-D, whereas the produced callus on media supplemented with 1 mg L^-1 2,4-D or 2 mg L^-1 NAA gave 5.43 and 2.36 ELS, respectively. It is well known that PGRs play important roles in plant cell division and differentiation. Nevertheless, PGRs that most widely affect organ regeneration, callus induction, or induce somatic embryogenesis are auxins and cytokinins (Nic-Can and Loyola-Vargas, 2016). In addition, auxin plays a central role in early and postembryogenic plant development (Cueva-Agila et al., 2016). Also, the combination of auxins and cytokinins was a prerequisite for indirect somatic embryogenesis of strawberry tree (Arbutus unedo) (El-Mahrouk et al., 2010).

Table 3. Growth of the regenerated haploid embryos of black cumin on MS medium without PGRs.

- **Fig. 3. Flow cytometric analysis based on median fluorescence intensity (MFI) of black cumin:** (A) diploid plant, (B) haploid plant regenerated through direct gynogenesis of unfertilized ovule culture, (C) haploid plant regenerated from callus of unfertilized ovule culture, and (D) tetraploid plant.
The ability for spontaneous diploidization varies, depending on many factors (Kiełkowska and Adamus, 2010). The peaks of the in vitro-regenerated haploids were observed in 1960 and 1829 MFI for direct and indirect haploid plants, respectively. The histogram of tetraploid plants was observed in 8470 MFI. Flow cytometry data are displayed in the form of a histogram of relative fluorescence intensity, representing relative DNA content (Dolezel and Bartos, 2005). Therefore, plants which have MFI with 1960 and 1829 are surely haploids when compared with diploid and tetraploid MFI. It is noted that MFI of haploid plants were near the value but not equal. The previous studies for using laser flow cytometry on sunflower (Helianthus annuus) of 13 diploids (2n = 34) showed variability in mean DNA content exceeding 27% and 48% among leaves from different nodes of plants of the open-pollinated variety and the inbred line, respectively (Michaelson et al., 1991). The variation of sunflower DNA content could be due to DNA-staining inhibitors effects (Price et al., 2000). Portugal and Waring (1988) mentioned that DAPI binds to adenine and thymine-rich DNA-sequence. Our results confirmed the production of haploids through in vitro gynogenesis. The reported results can be observed in 1960 and 1829 MFI for direct and indirect haploid plants, respectively (Couto et al., 2013; Dolezel and Bartos, 2005; Kielkowska and Adamus, 2010). They reported that flow cytometry has much higher relevance than any other proposed method for ploidy analysis of haploid regenerants.

The present study reported a simple effective protocol for in vitro production of haploid plants. Among several PGR treatments, 2 mg L⁻¹ 2,4-D was optimal of direct gynogenesis, whereas 1 mg L⁻¹ 2,4-D resulted indirect gynogenesis. Flow cytometry analysis confirmed the production of haploids through in vitro gynogenesis. The reported results can facilitate breeding programs of black cumin in which haploids can be produced in a short time frame to generate homozygous lines.

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