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Plant regeneration from hypocotyls of black carrot via direct somatic embryogenesis and determination of its genetic stability by RAPD and iPBS methods

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Abstract

A study was carried out to investigate plant regeneration from hypocotyls of black carrot (*Daucus carota* spp. *sativus*), an industrially and medicinally important plant via somatic embryogenesis, and the genetic stability of obtained plantlets with the random amplified polymorphic DNA (RAPD) and inter primer binding sites (iPBS) methods. Hypocotyl explants isolated from *in vitro* germinated seeds were incubated initially at Murashige and Skoog (MS) medium containing 0.5 mg/L or 1 mg/L 2,4-D for 2 weeks and then at hormone-free MS medium in order to obtain somatic embryos, which were observed after 15 days on the explants. Performed molecular analyses showed that all bands obtained from plantlets through micro-propagation were monomorphic. This protocol where black carrot was determined to be clonally reproduced through somatic embryogenesis may lead the way for further research regarding germplasm conservation and breeding studies.

Key words: Black Carrot, direct somatic embryogenesis, micropropagation, molecular markers, plant growth regulators

Introduction

Black carrot (*Daucus carota* ssp. *sativus* var. *atrorubens* Alef.) is a valuable vegetable not only due to its high nutritional value but also due to its industrial significance (Kamiloglu et al. 2015). It is the best coloring vegetable that can be used in place of synthetic food coloring by food industrialists due to the bright color it gives to foods it is added on, its pleasant taste, and high stability. It is being used as a natural material for food coloring in various products such as fruit yogurts, cakes, chocolates, and candies (Stintzing and Carle 2004). In the recent years, studies conducted using plant tissue culture methods that involve production of new tissues, plants or plant-related products from a whole plant, cells, tissues or organs in asceptic conditions and an artificial nutrition environment have gained significance (Gurel et al. 2013). One of these techniques, the method of somatic embryogenesis, allows obtaining embryos from somatic cells, and as a result, production of multiple plants. Somatic embryogenesis happens in two ways as direct and indirect. In direct somatic embryogenesis, somatic embryos develop from a single cell or groups of cells on the explant tissue without an intermediary callus stage. Direct somatic embryogenesis is a preferred approach for obtaining genetic stabilized regenerated plants (Khilwani et al. 2017).

As the visible morphological variation in plant culture studies aiming for clonal multiplication happens in much lower frequency than those at DNA level, studies towards the usage of molecular biology techniques in the analysis of genetic stability are prominent. The RAPD methods, which is one of the molecular marker methods for determining genetic polymorphism, is a technique that is used to detect variety among genetic sources, relationships among individuals in plant populations and in genetic mapping studies, which is inexpensive and preferred because of its time-efficient nature (Rafii et al. 2012; Paul et al. 2016).

Recently, another exceedingly universal and efficient molecular marker based on the conversed sequences of retrotransposons, i.e., interprimer binding site (iPBS), has been developed by Kalendar et al.

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(2011). Plant genomes include several transposons (Lee and Kim 2014). It has been reported in a number of studies that the activation state of transposons are stimulated by internal and external factors, and that there may occur great changes in the transpose on activities especially under stress conditions (Ito et al. 2016, Negi et al. 2016; Wang et al. 2017). The conditions applied in plant tissue culture studies may caused transposons to be activated by creating stress in plants (Azman et al. 2014; Paszkowski. 2015). As a result of genetic or epigenetic changes due to transposons during the culture, cells that are morphologically, physiologically or genetically different from each other may form and the obtained plant may carry different characteristics from the plants that is the source of explant (Larkin and Scowcroft 1981). The iPBS method is a fast, low-cost, and efficient molecular method applicable to plant breeding (Bayat et al. 2018; Mehmood et al. 2013). The objective of the present investigation was to study micropropagation of the black carrot plant via direct somatic embryogenesis and find out genetic stability of the obtained plantlets using RAPD and iPBS molecular markers.

Materials and methods

The Black Carrot (*Daucus carota* ssp. *sativus* var. *atrorubens* Alef.) plant was used for micropropogation. Murashige and Skoog (MS) nutrient medium containing 0.1 g/L myo-inositol, 30 g/L sucrose and 0.8% g agar (w/v) was used (Murashige and Skoog 1962). The pH of nutrient media was adjusted to 5.8 using 0.1 N HCl and 0.1 N NaOH. Seed germination studies were conducted at 24±5°C in dark and somatic embryogenesis studies were done under a 2000 lux white fluorescent lamp with a 16h/8h photoperiod in a plant growth room.

Somatic embryogenesis studies

To obtain the plantlets to be used as source of explant, black carrot seeds were left in 70% ethanol for 3 min, 0.5% NaOCl for 5 min for sterilization and then rinsed by leaving for 3 min in each of the 3 consecutive containers of sterile distilled water. The seeds were germinated in MS medium without any plant growth regulators. The hypocotyl parts of 7-day-old plantlets obtained from germinated seeds that did not contain apical meristem were cut in 0.5 cm lengths, and incubated for two weeks in petri plates containing MS medium 0.5 mg/L or 1 mg/L of 2,4-D. At the end of the two weeks, the explants were transferred to MS medium without any plant growth regulators and left for incubation for 30 days.

Determining genetic stability with RAPD methods

The DNA of the plants was isolated using the GeneJET plant genomic DNA purification mini kit (Thermo Sci.) based on the manufacturer's protocol. In RAPD-PCR studies, 8 primers (Operon Technology, Almeda, Canada) (F16, 015, 011, N12, K6, T16, P6, M6) were used (Table 1). The reaction mixture (10 µl) contained

Table 1. Nucleotide orders of primers used in RAPD studies

Primer	Sequence (5'-3')
OPF-16	GGAGTACTGG
OPO-15	TGGCGTCCTT
OPO-11	GACAGGAGGT
OPN-12	CACAGACACC
OPK-06	CACCTTTCCC
OPT-16	GGTGAACGCT
OPP-06	GTGGGCTGAC
OPM-06	CTGGGCAACT

1µl Taq DNA polymerase (Fermentas), 1.2 µl dNTP mixture (Promega), 1.5 µl 10 X PCR reaction buffer, 2 µl $MgS0_4$, 1 µl primer (Operon) and 20 ng genomic DNA. PCR reaction conditions are 35 cycles as 30 s at 94°C and 25 s at 94°C, 45 s at 35°C and 1 min at 72°C, and 5 min at 72°C for the last cycle. Products of PCR were run in a 1 % agarose-gel electrophoresis. The gel was stained with ethidium bromide and inspected under UV light.

Determining genetic stability with iPBS method

The DNA samples obtained from plant leaves acquired as a result of micropropagation were tested with 7 iPBS primers (Table 2) designed by Kalender et al. (2010). The DNA amplification procedure was carried out by changing the protocol defined by Kalender et al. (2010). The PCR reaction mixture (15 µl) contained 6 µl DNA (30 ng), 1.5 µl 10X PCR buffer, 1 µl primer, 1.2 µl dNTP mixture, 0.2 µl Taq DNA polymerase (GoTaq, Promega), 2 µl $MgCl_2$, 3.1 µl dH_2O. PCR studies were conducted with A Peltier thermal cycler (DNA Engine DYADTM 10 Bio-Rad, Hercules, CA, USA). PCR thermal cycles were; initial denaturation 94°C for 3 min; 35 cycles 94°C for 30s, 46-56°C

Table 2. Base sequences of primers used in iPBS-PCR and elongation periods

Primer	Sequence(5'-3')	Ta(°C)
2237	CCCCTACCTGGCGTGCC	46
2270	ACCTGGCGTGCCA	48
2391	ATCTGTCAGCCA	48
2393	TACGGTACGCCA	48
2394	GAGCCTAGGCCA	48
2395	TCCCCAGCGGAGTCGCCA	56
2398	GAACCCTTGCCGATACCA	56

Ta = Temperative

(changing based on primer; Table 2) hybridization temperature for 30 s, 72°C for 2 min elongation and 72°C for 5 min final elongation. The program was kept at 4°C at the end of the cycles. The obtained PCR products including 10 µl DNA, 1 kb DNA ladder were run in 3 % agarose-gel electrophoresis at 1XTBE buffer at 50 mA. The gels were imaged under UV light (G-box SYNGENE).

Results and discussion

The aim of this study was to ascertain the micropropagation protocol of the black carrot plant through direct somatic embryogenesis and to determine the plant's genetic stability. Black carrot sets seeds in the second year when grown in field conditions. It is difficult and laborious to collect, sort out and clean the obtained seeds. For this reason, its propagation with somatic embryogenesis has been investigated. Many studies reported that auxin application is necessary for embryo development from somatic cells (Ipekci and Gozukirmizi 2003; Guo and Zhang 2005; Deo et al. 2010). Auxins have different effects in different stages of embryogenesis; while they are necessary in the formation of cell blocks (initiation), they inhibit in the stage of plantlet development from cell blocks (development) (Komamine et al. 1992). In the present study, in order to obtain somatic embryos, 2,4-D has been preferred from among the auxin group plant growth regulators. Following the 14-day incubation of 0.5 cm long hypocotyl segments isolated from plantlets obtained in MS medium containing 0.5 mg/L or 1 mg/L 2,4-D, and the 30-day incubation in MS medium without any growth regulators, direct somatic embryo formation was observed on explants. It was observed that best somatic embryo formations were on hypocotyl segments in media containing 0.5 mg/L 2,4-D starting

Fig. 1. Somatic embryos developed from hypocotyl explants

with the 15th day (Fig. 1). Huynh et al. (2017) reported success of somatic embryogenesis in soybean with application of 2,4.D@40mg^{-1}.The obtained plantlets were transferred to new media in 2-week periods by dilution in order to allow them to develop better (Fig. 2).

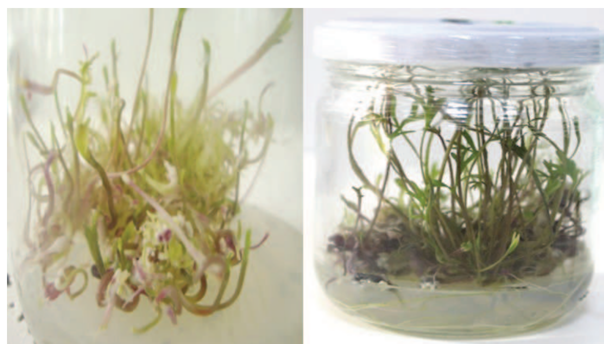

Fig. 2. Plantlets developed from somatic embryos

High auxin application used in the somatic embryogenesis method to obtain embryogenic cells that divide rapidly induces stress in plants and may lead to chromosome fragmentation or loss of chromosomes, and genomic modifications such as fraction or fusion, made easier by transposition events (Ozkul et al. 2016). Therefore, the amount of 2,4-D applied to obtain somatic embryos and whether the duration of incubation caused any genotypic changes were investigated by the RAPD and iPBS methods. The RAPD analyses of plantlets resulted by

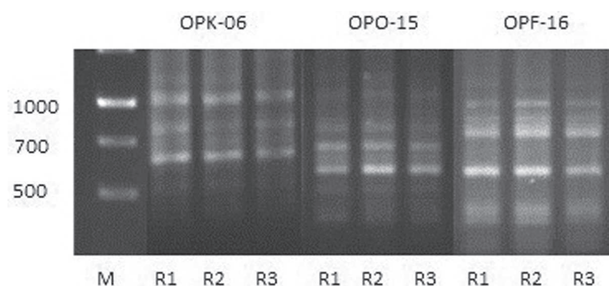

Fig. 3. Black Carrot RAPD band profiles; Primers (OPK-06, OPO-15, OPF-16), R1-3; Regenerated plants (R1-3); Marker (M)

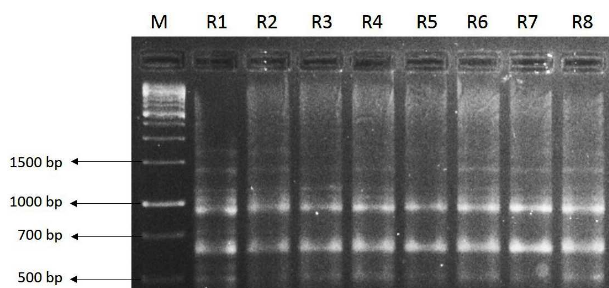

Fig. 4. Black Carrot iPBSband profiles with primer 2270; Regenerated plants (R1-8); Marker (M)

micropropagation and no polymorphism was observed in the obtained bands. Figures 3 and 4 show monomorphic bands provided by samples with RAPD primers. In literature, there are many studies which applied the RAPD analyses on various plants produced with plant tissue culture methods and in which monomorphic bands were observed (Fatima et al. 2012; Aslam et al. 2015; Patil and Bhalsing 2015; Abass et al. 2017). The result of the study echoes the study by Takeda et al. (2008) on the carrot plant obtained by a two-stage direct embryogenesis.

Several researchers, such as Al-Najm et al. (2016); Gedik et al. (2017) and Coutinho et al. (2018), have reported the high efficiency of the iPBS markers in identifying the high genetic diversity among plants. Studies in literature are newly emerging on the usage of this technique produced with plant tissue culture (Gozukirmizi 2014; Zahumenická et al. 2018). Genetic stability was also determined with iPBS as the second method black carrot plantlets were investigated with seven iPBS primers. Monomorphic bands were obtained in all of them. Figure 4 illustrates the monomorphic bands obtained with a 2270 iPBS primer. In this study, it was found that it is possible to produce the black carrot plant, which has the capability of seeding in the second year in field conditions, in a short time and a high quantity with the somatic embryogenesis method, which is a plant tissue culture method. It was concluded with the applied molecular techniques that the culture conditions did not lead to any genetic instabilities. There are a very few reports on the use of iPBS in plant tissue culture. The findings indicated that the protocol determined may be useful and may forward in making possible the seed production and plant output, independent of environmental conditions at the desired level and quality. Probably, the microproporation through somatic embroyogenesis of black carrot has been carried out for the first time.

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