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Development of a procedure for production of haploid plants through microspore culture of roselle (*Hibiscus sabdariffa* L.)

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ABSTRACT

Attempts to obtain new variants of roselle through conventional breeding were unsuccessful for 358 trials of (UKMR-3 × UKMR-2) and 63 trials of (UKMR-3 × accession 3). Roselle is an autotetraploid plant ($2n = 4x = 72$). Therefore the objectives of this study were to obtain new variants in roselle through the establishment of a plant regeneration system via microspore culture followed by detection of the ploidy level of regenerants based on their distinctive PCR patterns. The effects of different media and hormonal combinations were evaluated on microspore culture of roselle. This method was effective for callus induction, early-stage embryo development and subsequently efficient plant regeneration. Pretreatment of microspores at 4 °C and 35 °C for 3 days in the dark and without pretreatment, showed significant results on percentage of callus induction. Thirty regenerants were evaluated for their ploidy level using flow cytometry combined with propidium iodide and only 1 was detected as haploid, 4 mixploids and 25 diploids. Polymerase chain reaction (PCR) analysis using M13 universal primer (5′-TTATGAAACGACGGCCAGT-3′) showed the regenerated haploid plant having four unique bands at loci M13-01, M13-03, M13-04, and M13-06, which were absent in the microspore donor plant UKMR-1. To our knowledge, this is the first report of the development of haploid plants in roselle via microspore culture.

1. Introduction

Roselle (*Hibiscus sabdariffa* L.) is a dicotyledonous plant that belongs to the family Malvaceae. The plant grows annually and sometimes biannually reaching a height of 2–2.5 m. The leaves are deeply lobed, 8–15 cm in length and arranged alternately on the stem. Roselle can be found in almost all tropical countries such as Malaysia, Indonesia, Thailand, and in the Philippines (Rao, 1996; Chewonarin et al., 1999). In Malaysia, commercial planting of roselle began in the state of Terengganu in 1993 to replace tobacco and now it has spread to other states. The flower sepals are widely used for the preparation of herbal drinks, cold and warm beverages, and for making jams and jellies (Rao, 1996; Abu-Tarboush et al., 1997; Tsai et al., 2002; Akindahunsi and Olaleye, 2003).

Roselle has 72 chromosome numbers and categorized as an autotetralpoid ($2n = 4x = 72$) (Menzel and Wilson, 1963). Conventional hybridization of roselle is difficult due to its cleistogamous nature of reproduction (Allard, 1960). Improvement of roselle through conventional and mutation breeding has been conducted since 2006 (Mohamad et al., 2011) resulting in the release of new cultivars namely UKMR-1, UKMR-2, and UKMR-3, with cv. Arab as the parental cultivar. The three cultivars have been proven to exhibit high morpho-agronomic traits compared to cv. Arab and cv. Terengganu, contain high hydroxycitric acid (HCA) compared to *Garcinia atroviridis*, and also high in anthocyanins (Mohamad et al., 2011; Omalsaad et al., 2012). To complement the mutation breeding programme, generating genetic variants through microspore culture would be most beneficial. Currently there is no report describing methods for anther or microspore culture in *H. sabdariffa* L. while there is only one report on multiple shoot regeneration of roselle through shoot apex culture (Gomez-Leyva et al., 2008).

Hu and Kasha (1999) found that stress treatments have been shown to induce androgenic development in microspores of wheat (*Triticum aestivum* L.) cv. Chris via one of three different pathways A, B, or C. The A pathway is the continued karyokinesis of the vegetative nucleus (A-V), the generative nucleus (A-G), or both (A-VG)

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following the asymmetric first mitotic division. The B pathway is a symmetrical first nuclear division of the uninucleate microspores leading to two similar vegetative nuclei. The C pathway is the fusion and continued mitotic division of the vegetative and the generative nuclei resulting in the formation of callus or embryoids with a diploid chromosome number.

Haploids of higher plants can be distinguished from their diploids by using conventional cytological techniques and measurement of the DNA content using flow cytometry (Bohanec, 2003). This direct method of measurement of DNA content provides a rapid and simple option for large-scale ploidy determination as early as in the in vitro culturing phase. It also enables detection of mixploid regenerants and the determination of their proportion.

This study was completed to develop a protocol for the microspore culture of roselle with the final objective of generating genetic variants. The study included the identification of buds with microspores at the early uninucleate stage, establishing anther sterilization method, establishing and testing procedures and media for callus induction from the microspores, determining the predominant pathway of androgenic induction, shoot regeneration and adventitious root induction. Finally, the ploidy level of regenerants was determined using flow cytometry and variants obtained were compared with microspore donor plants using M13 universal primer (5′-TTATGAAACGACGGCCAGT-3′) (Ipor et al., 2007; Welsh et al., 1991). The use of M13 universal primer requires no prior knowledge of the genome that is being analyzed because it is present in most plants and can be employed across species. It is also known as arbitrarily primed polymerase chain reaction (AP-PCR), a variant of RAPD. As RAPD is based on the presence or absence of bands, there is a maximum of two alleles available at each locus. Therefore, they are not particularly diverse at the single locus level and so many loci can be uncovered with these markers. Thus, enough polymorphic loci can usually be found for most purposes.

2. Materials and methods

2.1. Plant materials

The seeds of four cultivars of *H. sabdariffa* L. [UKMR-1, UKMR-2, UKMR-3 and Accession 21(cv. Arab)] were sown in the field at Universiti Kebangsaan Malaysia (UKM). Field planting was chosen because earlier attempts to plant the cultivars in the greenhouse resulted in low plant vigor, less bud numbers and thus a limited microspores supply. Flower buds (0.8–1 cm in diameter) were collected from 8-week-old plants. The flower buds contained 98% uninucleate microspores (Fig. 1a) and the other 2% were binucleates (containing generative and vegetative nuclei) (Fig. 1b) confirmed by making squash preparation of the microspores based on the procedure of Bradley (1948). Microspore nucleates were observed under a light microscope (BX41TF-CCD) and documented by a digital camera (Olympus BX41). Flower buds of the four cultivars were placed in different plastic bags to avoid mixing and brought to the laboratory for culturing.

2.2. Sterilization procedure

The flower buds were washed under running tap water containing 5 drops of Tween 20 (detergent) for 1 h, surface-sterilized in 70% ethanol for 1 min and rinsed three times with sterilized distilled water. The buds were next soaked in 70% sodium hypochlorite for 20 min, rinsed three times with sterilized distilled water, then cut in the middle to expose the anthers. Anthers were placed in a sieve, soaked in 20% sodium hypochlorite for 5 min, and rinsed three times with sterilized distilled water. This was a compulsory step since the roselle anthers were sticky and highly infested with indigenous bacteria. Preliminary experiment indicated up to 85% bacterial contamination after 3 days of culture if this step was omitted. Viability of microspores after sterilization of anthers was determined by flourescein diacetate (FDA) staining (Dunwell, 1985) and observed under an inverted microscope (AXIOVERT 135) with UV attachment.

2.3. Preparation of media

For callus induction, liquid MS (Murashige and Skoog, 1962) medium containing same treatments as solid media was poured into each of 100 ml conical flasks. Media for callus induction containing various combinations of growth regulators were distributed into petri dishes with each petri dish containing 15 ml of medium. Media for shoot regeneration and root induction were distributed into 150 ml conical flasks with each flask containing 100 ml of medium. All media used were adjusted to pH 5.8 with 1 N NaOH or 1 N HCl solution prior to autoclaving at 121 °C and 1.03 kPa for 15 min.

2.4. Induction of callus

After sterilization, the anther wall was pricked following the slit technique of Dunwell (1985) to release microspores from the anther capsules into 50 ml liquid medium. Liquid medium containing the anthers were sieved through a nylon 500-μm pore size filter to separate microspores from the anther capsules and stamens. The average of the microspore diameter was 114.21 μm (Fig. 1e) and that of the anther capsules was 1372.5 μm (Fig. 1f). Subsequently, the liquid medium containing microspores was filtered through a 30-μm nylon filter to trap the microspores. The microspores were checked under the light microscope to ensure they were free of somatic cells. Micropores were cultured in petri dishes containing solid MS, Driver–Kuniyuki walnut (DKW) (Driver and Kuniyuki, 1984), Gamborg's B5 (Gamborg et al., 1968) and McCown's (McCown and Lloyd, 1981) media containing 3% sucrose. The media were supplemented with various combinations of α-naphthalene acetic acid (NAA) (2.5, 5 and 10 μM) and 2,4-dichlorophenoxyacetic acid (2,4-D) (2.5, 5 and 10 μM), either alone or in combination with 5 μM benzyl adenine (BA) for callus induction and pretreated at 35 °C for 3 days in the dark (Dunwell, 1985) followed by dark incubation at 25 °C for 60 days. MS medium with 10 μM NAA found to be the best for callus induction (data not shown), was next modified with changes in the microelements concentration (Pan et al., 2004) for improved callus proliferation from the microspores.

The effects of microelements concentrations (5× and 10×) were tested against the normal microelements concentration of MS medium (Table 1). The next step was assessment of pretreatments on callus induction, whereby microspores placed on modified MS medium with 10× microelements concentration and 10 μM NAA

Table 1
Effect of microelements concentration on callus induction from microspores of *H. sabdariffa* L. cv. UKMR-1 after 8 weeks of culture.

Microelements concentration[a]	% Callus induction ± S.E.[b]
1×	1.3 ± 0c
5×	6.3 ± 0.03b
10×	13.2 ± 0.02a

Microspore cultures were pretreated at 35 °C for 3 days, followed by dark incubation for 60 days.

[a] Modified on microelements concentration in MS medium with NAA (10 μM).

[b] Data are means ± SE; means in the same column followed by different letters are significantly different at $P \leq 0.05$ based on Duncan's multiple range test.