Investigation of the Effects of Temperature and Starvation Stresses on Microspore Embryogenesis in Two Tetraploid Roses (Rosa Hybrida L.)

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ABSTRACT

In this research work, the isolated microspore culture system in two rose cultivars i.e. ‘Apollo’ and ‘Amarosa’ was investigated. Important factors including isolation media (AB, B), AT3 induction medium with different carbohydrate sources (sucrose, maltose and glucose) and amino acids source (lactalbumin hydrolysate) were studied. Stress is an important factor that could de-differentiate and regenerate microspores to a whole plant. Carbon starvation and temperature (heat and cold) treatments as two important stresses alone or in combination with each other for various periods were evaluated on the induction of sporophytic divisions. A mixture of different developmental stages of microspores was used to initiate the cultures but the majority of them were at late unilayer stage determined by DAPI staining. For eliminating bacterial or fungal contaminants, buds were surface-sterilized by immersion in 70% ethanol for 15, 30, 60 Sec. and 3.5% sodium hypochlorite solution for 5, 10, 15 min prior to microspore isolation. The best result was observed when microspores were sterilized with sodium hypochlorite (%3.5) for 10 minutes. Two isolation media did not show significant difference on the viability of microspores. Among induction media, in cv. ‘Amarosa’, the highest viability was observed in AT3 induction medium supplemented by glucose. Induction media in ‘Apollo’ cultivar didn’t show significant difference in microspores viability. Combination of starvation (B medium) and cold (4°C) treatment for 3 days induced embryo formation in cv. ‘Amarosa’. Present investigation for the first time reports a protocol for embryogenesis induction in rose (Rosa hybrida L.) microspores.

Key words: Embryogenesis; Isolated microspore culture; Rose (Rosa hybrida L.); Starvation; Temperature; Stress

Abbreviations: DH: Doubled Haploid DAPI: 6-diamidino-2- phenylindole

Introduction

The roses (Rosa sp.), favorite ornamental plants, are among the most important floricultural crops in the world. Rose improvement is depended on crossings followed by selection among large population progeny. However, grafting is expensive and traditional breeding is a time consuming procedure (Fathi and Soner, 2009). Traditional breeding methods have limited impact in the field of rose breeding. One of the methods for rose breeding is hybridizing between commercial varieties and wild species for transition of some useful genes from wild species to cultivated varieties. However, existing different ploidy levels among parents is a big problem. To overcome this problem, dihaploid plants could be produced from commercial tetraploids via haploidy procedure, and then such dihaploids which are diploids would be able to be crossed by wild diploid species in order to make new cultivars.
Haploids are individuals whose genomic constitution is the gametic chromosome number of the species. The most advantage of using doubled haploids in plant breeding is the immediate achievement of complete homozygosity. In fact, the production of haploid/doubled haploid plants allows one to speed up breeding programs, improve selection efficiency, detect linkage and gene interactions, estimate genetic variance and the number of genes for quantitative characteristics, produce genetic translocations, substitutions and chromosome addition lines and facilitate genetic transformation (Snape and Simpson, 1981).

Isolated microspore culture is one of the most commonly used methods to produce doubled haploid plants. Microspore culture has some advantages over other techniques. Large quantities of microspores can be isolated, providing millions of potentially embryogenic single haploid cells (Touraev et al., 2001). Study the effects of medium components on microspore performance is also possible (Touraev et al., 2001). When microspore, single haploid cell, is exposed to stresses, such as low or high temperature, carbon starvation and colchicine, they de-differentiate and regenerate into whole plant (Shariatpanahi et al., 2006a). Optimal combination of culture conditions and stresses can switch normal gametophytic developmental pathway of isolated microspore to a sporophytic pathway and subsequently produce embryos and haploid/doubled haploid (DH) plants. The production of doubled haploids can be a new way for genetic studies, as well as for plant breeding (Hofer and Lespinasse, 1996). Some factors to be considered when optimizing an isolated microspore culture protocol are involving: growing donor plants, harvesting floral organs, isolating microspores, culture and induction of microspores, regeneration of embryos, and doubling of chromosomes if required (Ferrie and Caswell, 2010).

Guha and Maheshwari (1964, 1967) reported embryogenesis from pollen of Datura innoxia for the first time. Until now, microspore culture of Brassica (Pechan and Keller, 1988; Takahata and Kelle, 1991), Nicotiana (Benito Moreno et al., 1988; Kyo and Harada, 1986), Hordeum (Hoekstra et al., 1993; Kuhlmann et al., 1991) and Triticum (Touraev et al., 1996; Shariatpanahi et al., 2006b) have been particularly well studied (Touraev et al., 1997).

According to authors search, there has not been any report concerning production of doubled haploid plants via isolated microspore culture in roses, yet. For the first time, Hofer (2004) reported the induction of embryogenesis and plant formation from isolated microspores of apple (Malus domestica Borkh.) from rosacea family. Haploid callus has been obtained also through anther culture of sweet cherry (Seirlis et al., 1979; Hofer and Hanke, 1990; Germaná et al., 2004 unpublished). Haploid callus obtained from peach anther culture, but no plants were regenerated (Michellon et al., 1974; Seirlis et al., 1979; Hammerschlag, 1983). Kadota et al (2002) obtained two embryos by pear anther culture, cv. Le Lectier, but their origin was not established and plant regeneration was not obtained. Tabaeazadeh and Khosh-khui (1981) reported callus induction from anther culture of two species of the genus Rosa. The fact that they observed a high frequency of diplloid callus from tetraploid Rosa species supports the assumption that they were originated from microspores; however the calli could not be regenerated. Therefore their origin from microspores is under question.

In this research work, isolated microspore culture was studied in order to induce embryogenesis in some cultivars of roses (Rosa hybrida L.).

Material and methods
Plant material
Experiments were carried out at Agricultural Biotechnology Research Institute of Iran (ABRII) during the years 2014-2015. Rose cultivars ‘Apollo’ and ‘Amarosa’, obtained from rose germplasm collection of ABRII. Flower buds were collected when microspores were at different stages from mid to late unicellular stage of development. Determining the correct developmental stage of the microspores, were performed with 0.1 mg l⁻¹ 4’, 6-diamidino-2- phenylindole (DAPI) (Vergne et al., 1986). Different solutions for sterilization were investigated. The buds were surface sterilized by immersion in 70% ethanol for 15, 30, 60 sec. and 3.5% sodium hypochlorite solution for 5, 10, 15 min, then 3 rinses with sterile double-distilled water.

Isolation and culture of microspores
Anthers were stirred in 2 ml medium B or AB (see “Media composition”, below) with a magnetic stirrer in a small tube for 2-3 min at 250 rpm; the crude microspore population was then filtered through a 58-mm filter; and the resultant filtrate was washed two times with the same medium by centrifugation at 1200 rpm for 5 min. The microspore pellet obtained after the final centrifugation step was re-suspended in medium B (in Petri dishes of 6-cm diameter) or induction medium (see “Media composition”, below). During the induction phase, the cultures were kept at 25°C in the dark. Microspore viability was determined by fluorescein diacetate staining (Heslop-Harrison and Heslop-Harrison, 1970) immediately after isolation of microspores, starvation and heat/cold shock.

Media composition
Medium B for isolation and starvation was according to Kyo and Harada (1986). Medium AB was identical to medium B but contained 0.3 M sorbitol in addition to 0.3 M mannitol (Shariatpanahi et al., 2006b). For the induction of rose
microspore embryogenesis, the basal medium of AT3 (Touraev et al. 1996) was used with three different carbohydrates sources including maltose (90 g/l), glucose, and sucrose. All of these induction media were tested with or without amino acids source (lactalbumin hydrolysate). The pH of AT3 medium was adjusted on 6.2. The media were involved AT3, AT3 with lactalbumin hydrolysate (AT3-L), AT3 with glucose (AT3-G), AT3-G with lactalbumin hydrolysate (AT3-LG), AT3 with sucrose (AT3-S) and AT3-S with lactalbumin hydrolysate (AT3-LS).

**Carbon starvation treatment**

Isolated microspores were incubated in B medium as starvation medium at 4, 25 or 30°C for 3 days under dark conditions. The pH of starvation medium was adjusted on 7. Constant humidity was maintained in duration of experiment. After stress duration, isolated microspores were transferred to AT3 medium.

**Heat or cold shock**

Isolated microspores were incubated at 4°C as cold shock for 10 and 14 days and 30°C as heat shock for 1, 3, 5 and 7 days. Then, isolated microspores were transported to room temperature (25°C).

**Statistical analysis**

In each treatment, three 6- cm Petri dishes were used as 3 replications. The entire experiments were repeated at least for three times in two years. Microspore viability and multi-cellular formation was calculated 20 days after inoculation. Experiments were performed as factorial based on completely randomized design (CRD). Analysis of variance was performed using MSTATC software. Means were compared by the Least Significant Differences (LSD) method. Data transformation including arcsine square root was applied to normalize the distribution.

**Results**

**Microspores developmental stage and buds sterilization**

The developmental stage of microspores used for culture is critical for success and varies depending on the species. A mixture of different developmental stages of microspores used to initiate the cultures but the majority of them were at late uni-cellular stage determined by DAPI staining (Fig. 1). Present study showed that when calyx opened and a few petals were visible on the buds majority of microspores were at the late uni-cellular stage.

For eliminating bacterial or fungal contaminants, buds were surface-sterilized prior to microspore isolation. Three treatments of ethanol did not show a significant difference in microspore viability and the percentage of contamination was very high, so we did not continue this treatment. The highest viability was observed when microspores sterilized with sodium hypochlorite (%3.5) for 5 minutes treatment, but the least infection of microspore cultures was observed when microspores sterilized with sodium hypochlorite (%3.5) for 10 or 15 minutes (Fig. 2). It confirms the report published earlier by Ciner and Tipirdamaz (2002) in pepper.

![Figure 1. DAPI staining of the rose microspore in: a late uni-cellular stage b multi-cellular structures of microspores c embryo structure from isolated microspore](image-url)
Isolation and culture media

Two microspore isolation media including B and AB were evaluated in this article. Different media (B & AB) did not show a significant difference on microspore viability (Fig. 3).

Figure 3. The effect of isolation media on microspores viability

In ‘Apollo’ cultivar, seven induction media were tested. According to the results observed there was no significant difference among the media. But the highest microspores viability was observed in AT3-LG and AT3-LS.

Three media in cultivar ‘Amarosa’ showed a significant difference in microspores viability. These media were involving AT3-G, AT3-LS and AT3-L. In this cultivar AT3-G had more effect on microspores viability (Fig. 4). Ferrie et al (1999) demonstrated a species-dependent effect on microspore culture efficiency (Ferrie et al., 1999).
Stresses applied for reprogramming of microspores towards embryogenesis

Sugar Starvation

One of the most effective stresses applied to microspores for induction of embryogenesis is sugar starvation. Combination of starvation with temperature stress was evaluated. Starvation treatment in ‘Apollo’ cultivar didn’t show a significant difference on microspores viability and multi-cellular structures formation. In ‘Amarosa’ cultivar, starvation treatment for 3 days at 4°C induced embryos (Fig 1c), but analyzed data did not show a significant difference among the three used temperatures (4, 25 and 30°C).

Temperature stress

Stress induces embryogenesis in microspores without stress; microspores follow their normal gametophytic pathway to form pollen grains (Touraev et al., 1997). Two widely used stresses are heat and cold shocks. The results of experiments on the effect of different temperatures for the induction of sporophytic divisions and viability of rose microspores (cv. ‘Apollo’) are shown in figures 5 and 6. The highest frequency of multi-cellular structures was observed in 30°C treatment for 7 days. The highest microspore viability was observed in 30°C for 7 days, 4°C for 14 days and 25°C (fig. 6). The best conditions for the induction and further development of microspore-derived embryos were found to be the 30°C for 7 days and 4°C for 14 days. Different high/low temperatures didn’t show a significant difference in multi-cellular structures formation and microspore viability of cv. ‘Amarosa’.

Figure 4. The effect of induction media on microspores viability in cv. ‘Amarosa’

Figure 5. The effect of different temperature stress on multi-cellular structures formation in cv. ‘Apollo’
Figure 6. The effect of different temperatures on microspores viability in cv. ‘Apollo’

**Discussion**

**Microspores developmental stage and buds sterilization**

Buds or tillers are typically harvested when the microspores are at the uni-cellular to early bi-cellular stage (Ferrie and Caswell, 2010). Generally, the sensitivity of microspores in various developmental stages (late-unicellular to early–mid bicellular) to inductive stresses is highly different (Touraev et al., 2001). This may be due to the transcriptional status which is still proliferative at that time and not yet fully differentiated (Malik et al., 2007). After the pollen grains begin to accumulate storage reserves, they usually lose their embryogenic capacity and follow the gametophytic developmental pathway (Heberle-Bors, 1989; Raghavan, 1990).

Briefly (1-2 min) immersing plant material in ethanol (70%), followed by immersion in sodium hypochlorite (6% or less) with a drop of Tween for several minutes (up to 15 min), followed by several washes with sterile distilled water, is one of the most commonly methods for surface sterilization (Ferrie and Caswell, 2010). Our results showed that the best sterilization treatment for eliminating contaminants was sodium hypochlorite (%3.5) for 10 minute, in this treatment the least contaminants and the most microspore viability was observed (Fig. 2).

**Isolation and culture media**

There was no significant difference between two isolation media. Actually, AB is the same as to B medium but containing 0.3 M sorbitol in addition to 0.3 M mannitol (Shariatpanahi et al., 2006b). The induction medium is an important factor in the induction and progression of microspore embryogenesis (Hofer, 2004). Suitable, nutrient-rich medium and appropriate culture conditions must be provided for isolated microspores. Microspore culture media similar to tissue culture media need macro and micro-nutrients, vitamins and a carbohydrate source (Ferrie and Caswell, 2010). In ‘Apollo’ cultivar, no significant difference was observed among seven induction media for viability of microspores, but the highest viability was observed in AT3-LG and AT3-LS. Among the three media (AT3-G, AT3-LS and AT3-L) tested in cv. ‘Amarosa’, the highest microspore viability was observed in the AT3-LG medium (Fig. 4). Stresses applied for reprogramming of microspores towards embryogenesis

**Sugar Starvation**

Starvation treatment didn’t result in a significant difference in microspores viability and multi-cellular structures formation in cv. ‘Apollo’. Starvation treatment as a stress has been used in some species i.e. tobacco (Kyo and Harada, 1986), wheat (Touraev et al., 1996), rice (Ogawa et al., 1994), barley (Hoekstra et al., 1992), apple (Hofer et al., 1999) or in cultured anthers of rice (Raina and Irfan, 1998) and rye (Ma et al., 2004). During the starvation treatment, qualitative and quantitative changes in protein kinase activities were shown (Garrido et al., 1993). Higher temperatures and longer starvation treatments decrease the efficiency of embryo induction considerably.
Starvation treatment for 3 days at 4°C induced embryos in cv. ‘Amarosa’, but analyzed data did not show a significant difference among the three temperatures (4, 25 and 30°C) (Fig. 1c). Culture of excised anthers of nine Austrian winter wheat genotypes under starvation and heat shock conditions induced the formation of embryogenic microspores at high frequency (Touraev et al., 1996). Hofer (2004) found that the most effective treatment for the induction of microspore embryos in apple was starvation of microspores for 2 or 3 days at 4°C.

Temperature stress

Brassica microspore embryogenesis was induced by heat shock treatment (Duijs et al., 1992). In cv. ‘Apollo’ the highest frequency of multi-cellular structures was observed in 30°C for 7 days. In this cultivar, the highest microspore viability was observed in 30°C for 7 days, 25°C and 4°C for 14 days treatments (fig. 6). The best conditions for the induction and further development of microspore-derived embryos were found to be the 30°C for 7 days and 4°C for 14 days. Cold shock has been reported to induce microspore embryogenesis in species such as tobacco (Duncan and Heberle, 1976). In heat-stressed microspores, several HSPs (Heat Shock Proteins) are synthesized (Binarova et al., 1997; Segui-Simarro et al., 2003), such as HSP70 which inhibits apoptosis (Jaattela et al., 1998). In cold-treated anthers, the total content of free amino acids is increased, which might be conductive to an adaptation of microspores to the metabolic changes and embryogenesis induction (Xie et al., 1997).

Conclusion

Present investigation, for the first time reported the induction of embryogenesis from isolated rose (Rosa hybrida L.) microspores in in vitro condition. A mixture of different developmental stages of microspores was used to initiate the cultures but the highest regeneration was obtained from the microspores at the late uni-cellular stage which was determined using DAPI staining. Sterilizing the microspores with sodium hypochlorite (%3.5) for 10 minutes showed the highest viability and least infection. There was no significant difference between two isolation media. AT3-G was the best induction medium for cv. ‘Amarosa’. Starvation treatment for three days at 4°C could induce embryos in cultivar ‘Amarosa’, but analyzed data did not show a significant difference among the three starvation temperatures. The best frequency of multi-cellular structures was observed in 30°C treatment for 7 days. The best conditions for the induction and further development of microspore embryos were found to be the 30°C for 7 days and 4°C for 14 days.

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References


