Plant Tissue Cult. & Biotech. **26**(2): 219-230, 2016 (December) **PTC&B**

Somatic Embryogenesis from Anther, Whole Flower, and Leaf Explants of Some Grapevine Cultivars

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Key words: Cultivar, Embryogenic calli, Media, *Vitis vinifera*

Abstract

To investigate the influence of cultivar, medium, and explants on production of somatic embryogenic callus in grapevine (*Vitis vinifera*) an attempt was made. After callus production, calli were transferred into GS1CA medium for embryogenesis. In GS1CA medium, anther explant of 'Shahroodi' cultivar showed the highest potential for production of embryogenic calli. Results showed that whole flower explants did not produce any embryogenic calli. In addition, leaf explants of 'Red-Sultanina' and 'Flame Seedless' were cultured on MS containing 1mg 2,4-D, 0.1 mg BA, 1 g/l casein hydrolisate, 20 g/l sucrose and 7 g/l agar were able to produce embryonic calli. After three months, calli were transferred to the MS with different concentrations of BA (1, 2 and 3.5 mg/l) and IAA (2, 5 and 15 mg/l). Results showed that among cultivars and different hormonal treatments, the medium containing 5 mg/l BA and 2 mg/l IAA induced maximum embryogenesis in 'Flame-Seedless' calli.

Introduction

Grapevine (*Vitis vinifera*) is one of the most important fruit crops in the world and Iran in particular. The main center of diversity of *V. vinifera* is believed to stretch from Iranian Plateau to the South coast of the Black Sea and it is believed that Iran is among the primary areas of domestication and cultivation for this fruit (McGovern 2003, Terral et al. 2010). This fruit tree is grown in 307,721 ha in Iran, producing 2.15 million tons annually, ranking first among fruit crops in this country (FAO 2014). Due to the long history of grape cultivation, Iran has many

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different grape cultivars. In spite of many advantages, commercial grape cultivars are facing some drawbacks including vulnerability to adverse climatic conditions as well as susceptibility to pest and diseases.

To improve grape cultivars, traditional classic methods have been employed for many years. However, classical methods for improving of fruit trees have some disadvantages including long juvenile periods, inbreeding depression, and incorporation of many unwanted characteristics to the progeny (Krul and Worley 1977, Gary and Benton 1991).

Somatic embryogenesis is considered as the most efficient method, especially when it is necessary to regenerate plants using genetically modified cells (George and Debergh 2008, Olah et al. 2009, Parimalan et al. 2011). This method is also an efficient tool for rapid propagation, somatic hybridization and somaclonal variation (Xu et al. 2005).

Somatic embryogenesis is a process in which differentiated and mitotically quiescent somatic cells recuperate embryogenic potential and differentiate new viable embryos that resemble zygotic embryos by re-programing gene expression (Chen et al. 2012. Due to its high potential of regeneration, somatic embryos are the most frequently adopted regeneration method in grapevine for gene transfer (Martinelly and Gribaudo 2009).

The use of somatic embryogenesis and the regeneration of plants have been studied for several cultivated species (Cardoso et al. 2012, Mishra et al. 2012, Ramakrishnan et al. 2013, Zhang et al. 2014). For grapevine embryogenesis, different explants, media and plant regulators have been tried and according to the reports, successful embryogenesis about this plant depends on several factors including medium (Perrin et al. 2001), genotype (Olah et al. 2009), explants type (Gambino et al. 2007), and explant developmental stages at the beginning of culture (Vidal et al. 2009). Genotype is an important factor affecting successfulness of somatic embryogenesis in grape and successful protocols have been produced for several genotypes (Perrin et al. 2004, Gribaudo et al. 2004, Xu et al. 2005).

Although somatic embryogenesis has been studied in some grapevine genotypes, but the procedure is not well established for many important cultivars (Kikkert et al. 2005).

Working with new cultivars or even cultivars which have grown in different climatic and soil conditions, requires further research to optimize embryogenic callus production procedures to determine convenient explants, medium, hormonal balance as well as culture conditions. To do so, in the present study, we studied the effects of explant types, kind and level of plant growth regulators as well as incubation conditions on embryogenic callus production in well-

known cultivars of 'Red-Sultania', 'Flame-Seedless', and 'Perlette' as well as internally important cultivar of 'Shahroodi'. All of these cultivars are grown commercially in Iran, but they face problems which need improvement.

Materials and Methods

The inflorescence from *Vitis vinifera* L. cv. 'Flame-Seedless', 'Perlette', 'Shahroodi' and 'Red-Sultanina' were collected from the vineyard 10 - 12 days before anthesis with nearly impact flowers and anther color was transparent and some green (Fig. 1). In order to exert the chilling treatment, the inflorescences were kept in the sealed containers at 4ºC for 24 - 48 hrs. Young leaves were collected from new seasonal growth of 'Red-Sultanina' and 'Flame-Seedless' vines. The size of the leaves was in the range of 2 - 5 cm and they did not show any pest or disease symptoms.

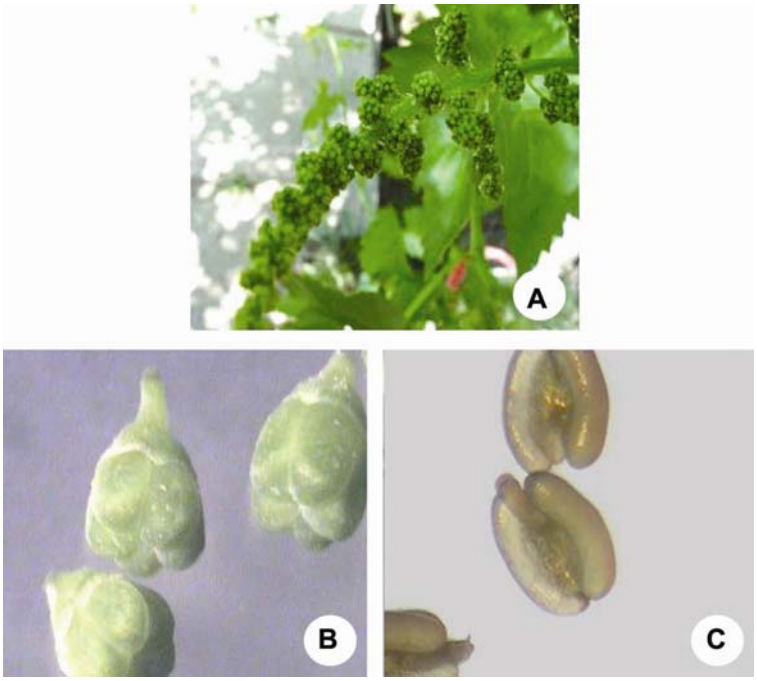

Fig. 1. Sampling stage and explants preparation. A. infloresence 10 - 12 days before anthesis. B. whole flower explants. C. anther explants.

In order to induce embryogenesis, calli produced from both explants were transferred to the GS1CA medium (NN macro, MS micro, B5 vitamin, Fe/EDTA, NOA (2-naohthoxyacetic acid) (2 mg/l), BAP (0.1 mg/l), IAA (3.2 mg/l). Temperature and light conditions were as in previous stage. Calli were

subcultured at four-week intervals. Data recording was carried out in the GS1CA medium from callus after two months.

The leaves were rinsed with water for 15 - 20 min, and then sterilized using 7% calcium hypochloride with 2 - 3 drops of Tween 20 for 10 min. Then, they were rinsed three times with sterilized distilled water. In order to control the probable bacterial infection, gentamycin solution (100 mg/l) was used for 30 sec, then, the explants were rinsed again with sterilized distilled water. Leaf explants were cut to 1 - 2 cm segments and were cultured in Petri dishes containing the same medium which was used for anther and whole explants. MS containing 1 mg/l 2,4-D, 0.1 mg/l BAP, 1 g/l casein hydrolysate, 20 g/l sucrose, and 7 g/l agar at pH 5.8 was used for callus induction. Cultures were incubated at 25ºC in dark and subcultured every 30 days.

Two months after culture and subculturing every 4 weeks, the calli with low growth, globular tissue and white to cream color were transferred to MS with 1, 2 and 3.5 mg/l IAA and 2, 5 and 10 mg/l BAP for rigorous induction of somatic embryos. The cultures were kept in dark at 25ºC for the first month and then light intensity was increased to 10 - 12 µe/m^2/s for the second month. In this type of callus, the embryos were observed in the primary stage during 2 weeks after transferring to foresaid light condition. During the next two weeks, light intensity reached to 45 µe/m^2/s gradually and as time passed, heart shaped embryos were observed in the surface.

Data were recorded during the experiment and categorized based on callus texture. Data analysis performed using SPSS and EXCEL (drawing the graph) software.

Results and Discussion

Immature anthers, whole flowers, and leaf explants produced callus, but some of the anther explants initiated callus and the rest became black and died.

Both PIV and Harst media induced callus, but their differences was apparent on embryogenesis of the calli to GS1CA medium. Lopez et al. (2005) divided the calli into two groups: type I, which contained globular white or pale yellow texture, and type II with completely soft texture and pale brown color.

Calli which were produced from anther and whole flower explants in Harst and PIV media, were studied from the point of callus type six weeks after their transfer to GS1CA medium. Calli were divided into one of the three types: watery, powdery and compact tissue, based on their tissue quality. Previous reports (Perrin et al. 2004, Lopez et al. 2005, Gambino et al. 2007) showed that embryo production is dependent on callus morphology. In this categorization,

watery calli (Fig. 2A) were those which did not have the ability of embryogenesis. These calli were dissipated with the scalpel. Powdery calli (Fig. 2B) were in white color and looked like a bulk. These calli were not embryogenic. Compact tissues (Fig. 2C) were those, which did not dissipate and were milky color. These tissues, in some of their sections, produced globular structure that showed to have the ability to be converted to heart and torpedo shape embryos (Fig. 2D). According to Marsoni et al. (2008), embryogenic callus are those which are translucent in color and more friable, whereas non embryogenic callus appeared to be spongy. Also Xu et al. (2005) observed higher frequency of somatic embryo in white, friable and crystal like callus than watery and loose ones.

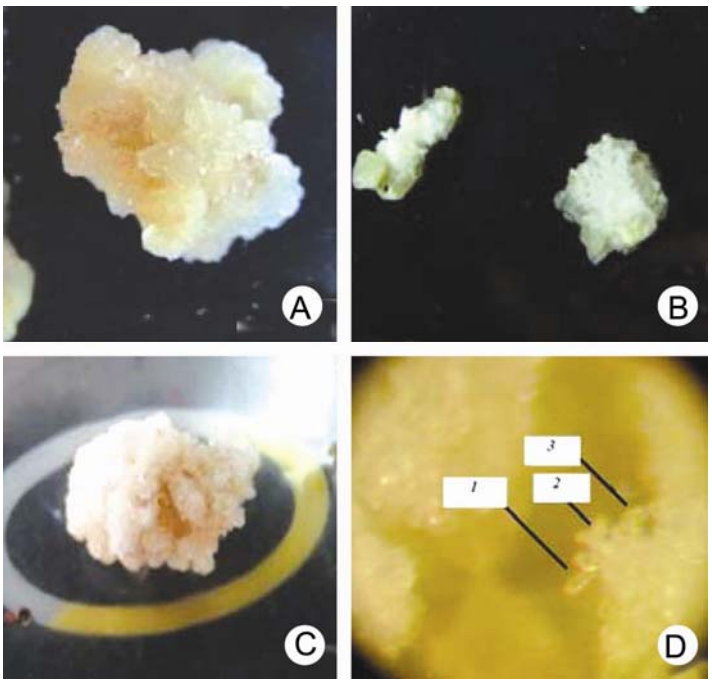

Fig. 2. Different types of calli: A. Callus with watery texture. B. Callus with powdery texture. C. Callus with compact texture. D. Eembryos in different stages (1. globular, 2. heart and 3. torpedo).

Results showed that, type of cultivar affects callus texture. Among cultivars, the most watery and powdery texture were recorded for 'Perlette' (43.3%) and 'Red-Sultanina' (37.5%). Therefore, they did not produce any embryogenic callus in GS1CA medium. 'Shahroodi' showed the highest percentage (52.6) of compact texture calli that is an assurance for the embryogenesis in the next steps, while 'Perlette' did not produce any embryogenic callus (Fig. 3).

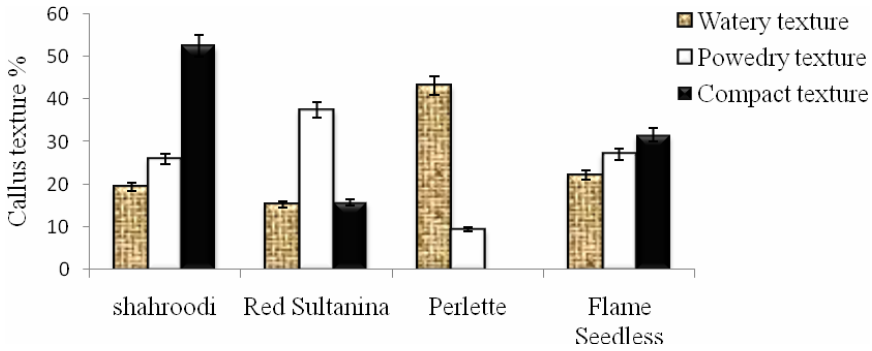

Fig. 3. Effect of cultivars on calli type produced from anthers and flowers.

The most important factor for inducing embryogenesis in callus, is the level of plant growth regulators in the culture medium. With regard to differences among the cultivars, it could be concluded that each and every cultivar has special need for exogenous growth regulators, which can be resulted in special response, when it establishes in an appropriate medium. These factors may affect the success of any cultivar to produce the embryogenic callus. Kikkert et al. (2005) reported that there were significant differences among genotypes for embryogenesis. This factor strongly affected induction of embryogenesis and the type of produced embryogenic tissue. The compact calli were produced from anther explants and had the embryogenic characteristics. These results are in agreement with Lopez et al. (2005) who reported the highest amount (52.5%) of embryogenic callus in cultivar 'Sugraone' from anther explants. Perl et al. (1996) reported that the amount of embryogenic callus in 'Suprior-Seedless' was 30.4%, while according to Mauro et al. (1986), the corresponding amount was 43.6% in 'Cabernet-Sauvignon'. In another similar work, Bouquet et al. (1982) reported 51.3% embryogenic calli formation from anther explants in *V. riparia*. However, Kikkert et al. (1997) showed a low frequency of embryogenesis (0.1 - 3%) in anther cultures of *Vitis* × *Labruscana*, after about one year. Xu et al. (2005) reported high per cent of somatic embrogenesis from immature ovules in some *V. rotundifolia* and *V. vinifera* cultivars. These results may confirm the idea that genotypes as well as species are important factors for embrogenic callus formation in grapevine.

Anther explants found to be mostly successful for producing the compact texture callus, and all of these calli converted to embryo in three 'Shahroodi', 'Red-Sultanina', and 'Flame-Seedless' cultivars. However, 'Perlette' did not produce any embryogenic callus. Whole flower explants in all the three cultivars did not produce embryogenic callus and most of their calli were powdery or

watery (Fig. 4). Generally, the embryogenic calli had low growth rate, compact texture, white to yellow color and globular shape. In our work, these types of calli (EC) were obtained only from anther and leaf explants. Direct contact of anther explants with medium surface has probably a prominent role on embryogenic callus production. Since, the anther explants do not have perianth tissue (sepal, petal, etc.), they established more effectively in the medium compared with the whole flower that had such organs. Therefore, in this trial, anther was the most suitable explants in the tested cultivars for producing embryogenic callus. Gambino et al. (2007) reported that the big size of whole flower explants may increase lag phase in response to growth regulators, and might be the reason of delay in production of embryos. They also observed that callus from whole flower culture in 'Chardonnay', 'Muller Thurgau', 'Grignolino' and '110R' had potential to produce embryogenic callus, but the same explants in cv. 'Brachettog. l' did not produce any embryo. Nevertheless, it seems that medium, environmental conditions and cultivar response were not set completely in our work for whole flower explants and it needs further investigations.

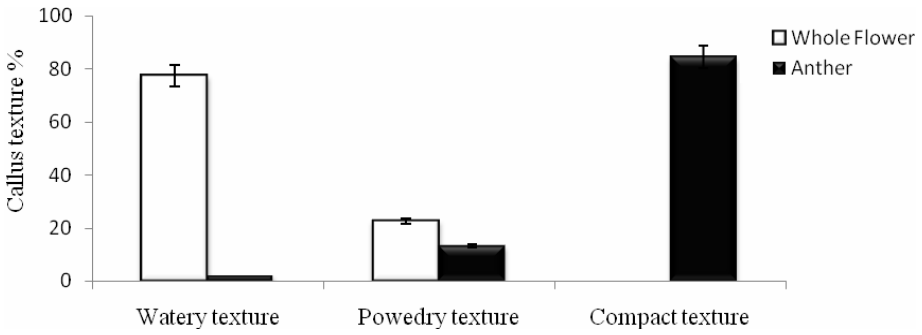

Fig. 4. Effect of explants on type of callus (watery, soft and dispersible).

In another trial, leaf explants in two cultivars, namely 'Red-Sultanina' and 'Flame-Seedless' were examined. The texture of callus from these explants was similar to those of anther calli (Fig. 5). This callus was assessed for embryo production in MS with 1, 2 and 3.5 mg/l IAA and 2, 5 and 10 mg/l BAP. The χ^2 test showed that the effects of cultivar was significant at 0.01 level for producing somatic embryos ($\chi^2 = 9.39$, $p = 0.01$).

Results from leaf explant indicated that, compared to 'Red-Sultanina'(31.4%), 'Flame-Seedless' showed better response (68.6%) for embryogenesis in the media for this explant (Fig. 6). The effect of culture media on embryogenesis might be due to balance of growth regulators of tissue as well as their external treatments.

Das et al. (2002) observed that 'Pussa-Seedless' in MS containing 1 mg/l IAA and 1 mg/l BAP produced the most embryogenic callus among the other cultivars ('Beauty-Seedless', 'Pussa-Seedless', 'Perlette' and 'Nashik'). In another attempt, Passos et al. (1999) were able to produce callus from the leaf explants in some grape cultivars, but those calli did not produce any embryo.

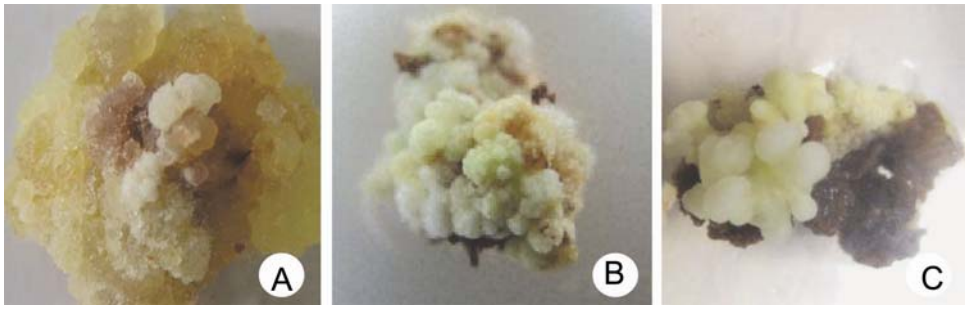

Fig. 5. Type of calli obtained from leaf explants. A. Normal callus in initiation medium. B. Embryogenic callus in dark condition. C. Embryo formation in light condition.

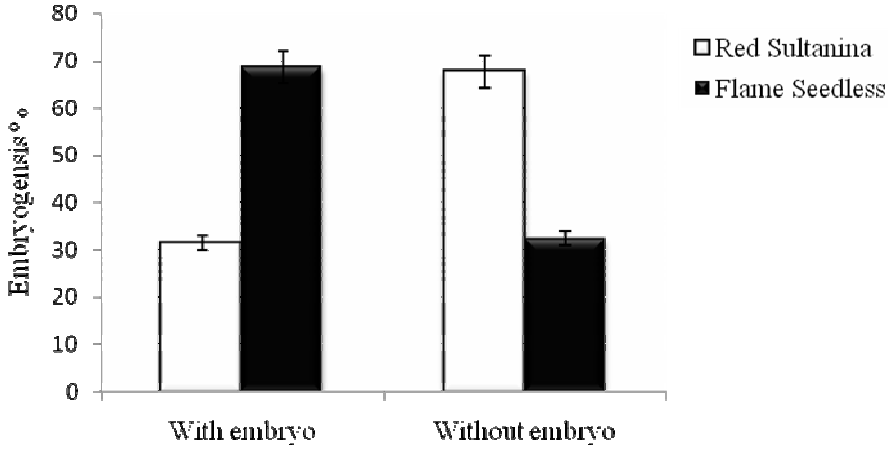

Fig. 6. Effect of cultivar on embryogenesis.

To study the effects of growth regulator treatments on somatic embryogenesis in the leaf explants, another trial was carried out with nine hormonal complex (BAP: 2, 5 and 10 mg/l, IAA: 1, 2 and 3.5 mg/l) in MS. Effect of treatments was significant ($\chi^2 = 28.46$, $p = 0.01$). Among the hormonal treatments, the medium containing 2 mg/l IAA and 5 mg/l BAP showed to be the optimal medium for embryogenesis. However, media with the same amount of IAA but different BAP concentrations did not induce embryogenic callus (Fig. 7). Combination of 2,4-D and BAP for production of callus from leaf explants was

investigated by workers (Robacker 1993,Torregrossa et al. 1995), and Matsyta (1992) produced somatic embryos using 2,4-D in leaf explants.

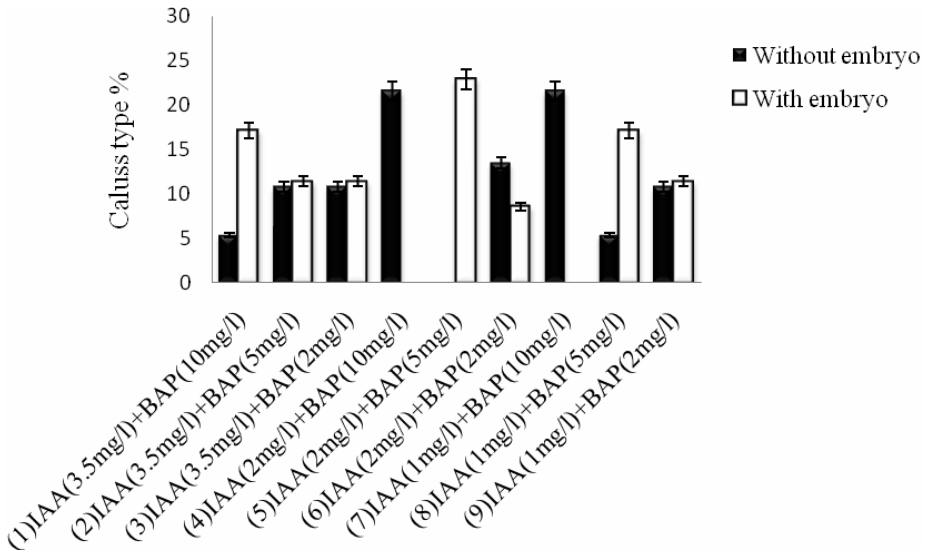

Fig. 7. Effect of different hormonal complex on embryogenic callus induction.

Lopez et al. (2005) working with 'Surgraone' and 'Crimson-Seedless' showed that embryogenic callus production is directly related to BAP concentration in the medium (1.3 mg/l). Effects of different concentrations of BAP and 2,4-D on the embryogenesis in the leaf explants were studied by Das et al. 2002. Although IAA (0.1 mg/l) and BAP (1.5 mg/l) have been reported to induce embryogenic callus in grape (Popescu 1996), the use of NN medium supplemented with IAA (1.7 mg/l) and BAP (2 mg/l) did not induce the formation of somatic embryos in anther-derived callus (Salunkhe et al. 1999). Matsuta and Hirabayashi (1989) reported that the lack of either 2,4-D or BAP, or both, in the medium is known to be detrimental for induction of somatic embryos in *V. vinifera* cell lines. Our results indicated that only the MS supplemented with 0.1 mg/l 2,4,-D and 1mg/l BAP induced the embryogenesis in the leaf explants which is in agreement with Popescu (1996) and Matsuta and Hirabayashi (1989). Results of another work on grape cv. 'Seyval-Blanc', the NN medium with 4 mg /l NOA and 0.9 mg/l TDZ showed the highest percentage of embryogenic callus production (Reustle et al. 1995). Results of present study revealed that, applied hormonal treatments (IAA 2 mg/l and BAP 5 mg/l) were very effective in embryogenesis in 'Red-Sultanina' and 'Flame-Seedless'. It can also be concluded that internal level of hormones in

any cultivar and its interaction with applied external hormones are very effective in embryogenic callus production.

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