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Somatic embryogenesis from leaf & petiole explants of some *Rosa hybrida* L. cultivars

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Abstract

In this study, somatic embryogenesis of four *Rosa hybrida* L. cultivars, including BlackMagic, HotLady, Audio and Eldorado, from leaf and petiole explants in invitro condition was investigated. Explants were taken in April and cultivated in MS medium. A combination of 2,4-D+BA and NAA+KIN were applied for callus induction. For somatic embryogenesis the ½ MS medium containing hormonal combination of 2,4-D, BA, NAA, KIN and GA_3 were used. The highest callus production was obtained from petiole explant, Audio cultivar and hormonal combination of NAA+KIN. The highest and lowest percentage of embryogenesis were manifested in HotLady and Eldorado cultivars, respectively. A hormonal treatment consisting of 0.5mg/l BA+0.3mg/l 2,4-D led to the highest rate of embryogenesis. Petiole explants led to a statistically significant ($p<0.05$) greater somatic embryogenesis.

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Introduction

Rose is considered one of the most economically invaluable flowers. Traditionally, it is propagated through root cutting or grafting, both of which pose great economic burden. In addition, they necessitate a great deal of effort and human labor. On the other hand, genetic breeding in flowers is restricted by the polypoloid phenomenon and heterozygosity. Remarkable achievements in propagation and regeneration of a variety of rose species in the invitro conditions have been reported. Somatic embryogenesis has been indicated as a potential invitro technique for rapid vegetative propagation and breeding of some rose species. In 1958, somatic embryogenesis was initially reported in carrot. According to Robert (1995) somatic embryogenesis in rose became an option in 1990.

Various reports on the somatic embryogenesis of rose from different plant sections have been reported. leaf callus explants of R.hybrida cvs. Domingo and Vicky Brown (De wit *et al.*, 1990), and R.Chinensis minima cv. Baby Katie and R.hybrida cv. Carefree Beauty (Hsia and Korban, 1996), immature leaf and stem segments of R.hybrida cv. Landora (Rout, 1991), invitro mature leaf of R.hybrida cv. Soraya (Kintzios *et al.*, 1990), anthers, petioles, receptacles, leaves of R.hybrida cv. Meirutal (Aren *et al.*, 1993), root of R.hybrida cv. Moneyway (Marchant, 1996), petiole of R.hybrida cv. Arizona (Robert *et al.*, 1995), immature embryo of R.rugosa (Kunitake *et al.*, 1993) and filament of R.hybrida cv. Royalty (Noriega and Sondhl, 1991). A variety of factors including non-responsiveness of cultivars to the application of somatic embryogenesis induction material (Kintzios, 1999; Murali, 1996), low induction rate of the embryogenic tissue (Rout, 1999; Hsia and Korban, 1996) and low frequency of germination (Sarasan, 2001; Rout, 1999) prevent somatic embryogenesis of the rose flower. Therefore, successful regeneration of rose hybrida is restricted to low cultivars. In addition, further studies on the improvement of the quantity of resomatization from somatic tissues of other Rose cultivars seems mandatory (Estabrooks *et al.*, 2006). In this study, the effect of a variety of factors including the cultivar type, growth regulators and the source of the explant on different stages of callus induction and somatic formation of the embryo in different Rose cultivars have been investigated in order to optimize somatic embryogenesis of this plant.

Material and methods

Stems containing Leaves of the two cultivars, BlackMagic and HotLady, were collected from the greenhouse of the horticulture department of Karaj Agriculture faculty. Seemly, Audio and Eldorado specimens were collected from the research farm of Jahad Daneshgahi of Karaj faculty. Specimens were taken to the lab; after removing the leaves and petioles from the stems, explants were exposed to multiple aseptic agents. At first, explants were placed under free water stream for 20 min with a few drops of dishwashing detergent applied. Thereafter, explants were rinsed in ethanol 70% for 50 seconds under laminar flow hood, after which they were treated with sodium hypochlorite(Naocl) 1% with 3 drops of tween 20 for 15 minutes. Explants were then washed with sterile distilled water for 3 times. MS basic culture medium was used for callus induction; 30g/l sucrose and 8g/l agar agar were added to the medium. After adding growth regulators, culture medium were adjusted to PH 5.8, then culture medium was sterilized in autoclave (Temp 121°C, 1 atm pressure for 20 min). Growth regulators including BA(2.3,2,1), 2,4-D(3,2.3,1.15), KIN(2.3,2,1) and NAA(2.5,2,1) were applied (hormonal values are in mg/l). Various treatments consisting of four R.hybrida L.cvs, twotypes of explants (leaf and petiole) and different compounds of growth regulators were used. Six pieces of leaf explants ($1cm^2$) in abaxial position and petioles (1cm) in horizontal position were placed. For embryogenesis induction, the half strength MS medium with growen regulators including BA(1,0.5)+2,4-D(0.3); BA(1,0.5)+GA_3(1); BA(1,0.5)+NAA(0.01)+GA_3(0.1); Kin(1.1,0.11)+NAA(0.05) were used (hormonal values are in mg/l). All cultures were incubated under a 16h photoperiod provided by cool-white fluorescent light (40-50 $\mu molm^{-2}s^{-1}$) except callus induction which was

conducted in the dark. with 24°c and 20°c daytime and night temperature, respectively. Each treatment for callus and embryonic callus induction was replicated four and tree times, respectively. Each replication consisted of a single Petri dish (100×15) containing 25ml culture medium. Experiments were performed as completely randomized factorial design. Results were described in percentages. Analysis of Variance were measured by SAS, MSTAT software and Duncan's multiple range test. Graphs draw were used by Excel software.

Results

Effect of 2,4-D+BA and NAA+KIN on callus induction

In this study, the greatest amount of callus induction was accomplished by applying a hormonal compound consisting of 2,4-D+BA in which the concentration of 2,4-D was highest and BA was minimally applied (the minimum concentration). on the other hand, lowest concentration of 2,4-D yielded the least rate of callus formation. the combination of BA+2,4-D hormonal treatments resulted in condensed callus formation with long durability, conversion into embryos was highly likely.

Table 1. The results of analysis of variance on percentage of callus induction in R.hybrida.

SV	df	MS
Cultivar	3	36514.14**
Hormone	17	4576.3**
Explant	1	14071.89**
Cultivar×hormone	51	1137.61**
Cultivar×explant	3	3514.28**
Hormone×explant	17	420.23**
Cultivar×hormone × explant	51	797.59**
Error	432	-
CV	-	12.37

** significant at 1% level.

With respect to other hormonal compounds, the highest frequency of callus formation was achieved with high concentrations of KIN+NAA. A reduction in callus formation was observed when the concentration of both hormones were reduced(Chart1). A positive correlation between the concentrations of these two hormones were observed at this state. Callus receiving NAA+KIN hormonal compounds had a short life duration with low density. In this study, different types of callus high density to like-cloud, cream to light yellow and brownish yellow in color, compact to tender and juicy were observed on explants (Fig 1 B-E). The color of callus appeared white to light yellow with good density in the presence of 2,4-D+BA compound, while it appeared light brown with low density and like-cloud, under the presence of KIN+NAA which rooted in the majority of cases.

With respect to the type of cultivar, the Audio cultivar achieved the highest percentage (67.62%) of callus formation, while HotLady yielded the lowest rate(36.12%). Petiole explants obtained a significantly greater callus formation. Regarding hormonal treatment, the compound containing 2.5mg/l NAA+2mg/l KIN resulted in the highest rate of callus formation, whereas the compound consisting of 1.15mg/l 2,4-D+2mg/l BA yielded the lowest rate of callus formation (75.4% vs 37%)(Chart 1). As indicated by variance analysis, the effect of all factors, genotype, hormonal compound, explants and effects of interaction between them was significant ($p<0.01$)(Table 1).

Effect of PGR$_s$ on somatic embryogenesis

In this study, by using various growth regulators, the highest frequency of embryogenesis was obtained in

the HotLady cultivar (30%) from explants treated with 0.5mg/l BA+1mg/l GA_3 and 0.5mg/l BA+0.3mg/l 2,4-D(Chart 2). In the Eldorado cultivar, embryogenesis was achieved only by hormonal treatment containing 1mg/l BA + 0.3mg/l 2,4-D. A hormonal combination consisting of 1mg/l KIN+0.05mg/l NAA in HotLady and BlackMagic cultivars yielded a 22% somatic embryogenesis, nonetheless this hormonal compound was ineffective in the two other cultivars. Overall, in this study HotLady cultivar yielded a 17.64% somatic embryogenesis, followed by Audio, Black Magic and Eldorado with 6.7%, 4.04% and 1.11% embryogenesis, respectively (Fig 1H-I). Overall, HotLady responded to all kinds of hormonal treatments, followed by Audio, in which embryogenesis was accomplished by the majority of treatment(Chart 2). According to variance analysis, Cultivar, explant, hormonal treatment and the correlation between hormonal treatment and the explant type, as well as between hormonal treatment and the cultivar, and cultivar with explant type were significant ($p<0.01$) (Table2).

Table 2. The results of analysis of variance on percentage of somatic embryogenesis induction in R.hybrida.

SV	df	MS
Cultivar	3	2451.57^{***}
Hormone	7	281.05^{***}
Explant	1	998.92^{***}
Cultivar×hormone	21	384.64^{***}
Cultivar×explant	3	350.85^{**}
Hormone×explant	7	91.93^{ns}
Cultivar×hormone× Explant	21	124.92^{ns}
Error	126	-
CV	-	13.55

** significant at 1% level, *** Significant at 0.1% level

^{ns} non significant.

Discussion

In this study, no callus was produced in the control group or those who didn't receive growth regulators (Fig 1A). Our findings were in the same line with previous studies conducted by Xiangqian Li *et al.*, (2002) and Estabrooks *et al.*, (2006). The greatest rate of callus formation was associated with the highest auxin concentration; furthermore, reduction in auxin concentration despite high cytokine levels decreased callus formation rate, which was in accordance with the findings of Xiangqian Li *et al.*, (2002). They yielded greatest callus formation rate using 11.3µmol/l 2,4-D in Carefree Beauty and Grand Gala cultivars. They indicated that increase in 2,4-D levels in the culture medium led to a significant reduction in frequency of callus induction. Kim *et al.*, (2003) indicated that when leaf explants are cultured on a culture medium with the least concentration of NAA+2,4-D (0.1 mg/l), low rates of callus induction were observed which corresponded with our findings. In addition, their reports indicated that higher 2,4-D concentration leads to the formation of a watery and extremely friable callus. In the present study, same results were yielded. In addition, a white and compact callus was generated by applying this hormone. The NAA+KIN hormonal compound led to the generation of a huge white callus which started browning after four weeks. After two weeks of callus formation, rooting of the calluses were observed (Fig 1F), which were in the same line with the results of Kim *et al.*, (2003). They reported rooting with different concentrations of NAA. Xiangqian Li *et al.*, (2002) emphasized the necessity of the 2,4-D treatment with or without other growth regulatory compounds in the in vitro conditions for initiating somatic embryogenesis. However, Kintzios *et al.*, (1999) observed a negative effect of 2,4-D administration for callus induction, which was proposed to be due to the

nonjuvenility of explants.

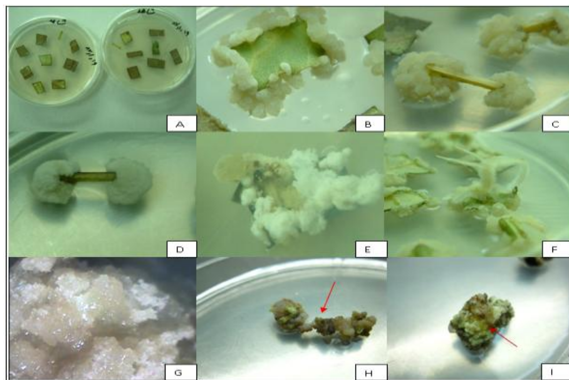

Fig. 1. A-I callus induction, embryogenic callus, somatic embryogenesis. A control explant. B-F callus induction. B leaf callus, C petiol callus, D compact callus, E like-cloud callus, F rooting callus, G embryogenic callus, H-I somatic embryogenesis.

Kim *et al.*, (2003) to be achived callus grayish-white and friable by treating with 2,4-D. No embryo or embryo-like structure was observed on any of the induced callus. Among the three different concentrations of 2,4-D applied, the highest rate of callus induction was observed on a 13.6µmol/l culture medium, followed by 10.4µmol/l. Xiangqian Li *et al.*, (2002) detected the highest frequency of callus induction on a culture medium with 11.3µmol/l 2,4-D concentration. Explants which didn't respond to cultures were necrotized.

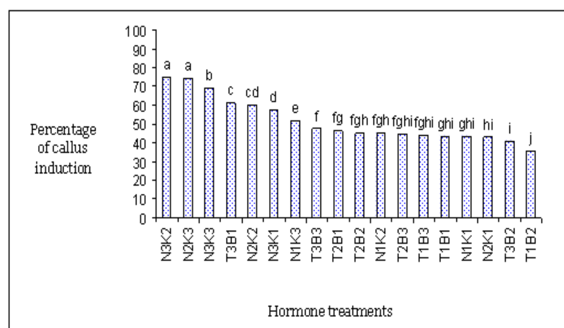

Chart 1. Comparison of callus Induction percentage and hormonal compounds. Means with the same letter are not significantly different at 5% level of Duncan test.

Xiangqian Li *et al.*, (2002) revealed that Callus inducedon leaf tissue was larger in size than that on the petiole, and when transferred to a basal medium in the absence of or at low levels of PGRs, the callus developed somatic embryos. In the study conducted by Kintzios *et al.*, (1999), better callus induction was observed in leaf explants in comparison with stem. Furthermore, it was indicated that the age and location of the leaf had no significant effect on culture reaction In contrast, callus formed on petiole explants of the Eldorado cultivar was significantly greater; however, difference in callus induction wasn't significant between the two types of explants. (Noriega&sondhl, 1991; kintzios *et al.*, 1999), reported that an auxin/cytokinin combination was essential for the induction of embryonic callus in rose tissue. The positive effect of KIN application on the callus growth and somatic embryogenesis has been previously described by (De wit *et al.*, 1990; Kunitak *et al.*, 1993). In BlackMagic and HotLady cultivars, 22-23% embryogenesis was achieved by the application of 4.6µmol/l KIN+0.27µmol/l NAA. During the present study, 1.66-10% embryogenesis occurred by treating the cultivars with 0.46µmol/l KIN+0.22µmol NAA which was in the same line with the findings of De wit *et al.*, (1990). A 30% embryogenesis rate was accomplished in the HotLady cultivar by using a combination of 2.9µmol/l GA_3+2.3 µmol BA. However, embryogenesis didn't occur in other cultivars by applying this treatment. Xiangqian Li *et al.*, (2002) introduced adding 2.9µmol/l GA_3 to either TDZ or BA containing media at any level has improved the frequency of somatic embryogenesis. GA stimulatus the production of numerous enzymes, notably α–amylase, in germinating cereal grains (Davis, 1995).

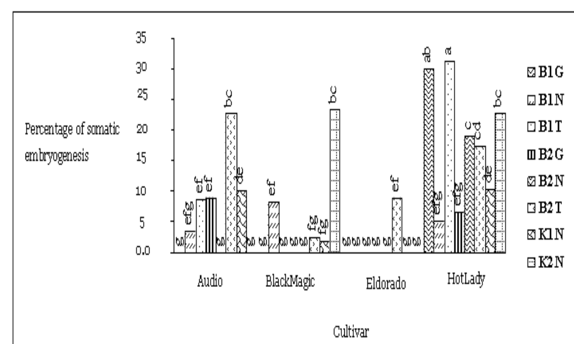

Chart 2. Comparison of somatic embryogenesis induction percentage and hormonal compounds. Means with the same letter are not significantly different at 5% level of *Duncan* test.

In the study conducted by Xiangqian Li *et al.*, (2002)

the highest frequency of somatic embryogenesis was yielded by the application of 2.9μmol/l GA_3+2.3 μmol/l TDZ, which indicated the positive role of GA_3 on somatic embryogenesis. However, the aforementioned hormonal treatment was ineffective in the remaining three cultivars. Therefore, it is not recommended as a single hormonal factor for rose cultivars; however, it can be one of the factors employed.

Difference between genotype reactions in somatic embryogenesis among various R.hybrid cultivars was described by (Hsia&Korban, 1996; Marchant, 1996). Kim *et al.* (2003) introduced genotype as a significant factor influencing explant embryogenic differentiation in cultivar, since only two of five investigated cultivars responded positively to somatic embryogenesis treatment. De wit *et al.*, (1990) by applying 1mg/l KIN+0.05mg/l NAA in seven rose cultivars, in two cultivars Doming and Vicky Brown yielded 3.5% and 1.5% embryogenesis rate on leaf explants. In the present study, out of the four rose cultivars only one achieved embryogenesis by one hormonal compound. Despite embryogenesis with some hormonal compounds, differences were non significant. The results revealed that genotype has the most important role in somatic embryogenesis. accordingly, multiple cultivar types are needed to optimize the outcome.

Abbreviations

BA 6-benzyladenine; 2,4-D 2,4- dichlorophenoxy acetic acid; GA_3 Gibberellic acid; KIN N-(2-furanylmethyl)-1H-purine-6-amine; MS Murashige and Skoog (1962); NAA 1- naphthaleneacetic acid; TB 2,4-D+BA. -NK NAA+KIN.

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