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Optimization of *In vitro* Propagation of *Rosa hybrida* L. Cultivar Black Red

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Abstract: An efficient protocol for *in vitro* micropropagation of *Rosa hybrida* was established using single node segment. The effect of different combination of plant growth regulators thidiazuron e.g. N6-benzyladenine (BA), Tthidiazuron (TDZ) naphthalene acetic acid (NAA) and Indole butyric acid (IBA) in MS medium on shoot and root initiation were tested. The most suitable medium for shoot and root proliferatin was T5 medium "MS+ IBA (0.1 mg l^{-1}), BA (5 mg l^{-1}) + sucrose (40 g l^{-1}). High concentration of auxin resulted in browning and necrosis of the explants. Acclimatized plants were transferred to the glasshouse and 70% of them survived.

Key words: *Rosa hybrida* · *in vitro* · propagation

INTRODUCTION

Propagation of plants through tissue culture has become an important and popular technique to reproduce crops that are otherwise difficult to propagate conventionally by seed and/or vegetative means. Specialized and mature cells are manipulated to give rise the multiple copies of the parent plant under optimum aseptic environmental conditions and appropriate stimuli. It offers many unique advantages over conventional propagation methods such as rapid multiplication of valuable genotypes, expeditious release of improved varieties, production of disease-free plants, non-seasonal production (a round the year), germplasm conservation and facilitating their easy international exchange. The wide-spread application of tissue culture has a few limitations such as cost of production, choice of crops restricted to species with acceptable micropropagation protocols, reproducibility of protocols [1].

There are more than 20,000 commercial cultivars of rose "the most important commercial crops" which collectively are based on only 80 of the approximately 200 wild species in Rosa [2]. Tissue culture or more specifically micropropagation of *R. hybrid* cultivars and *Rosa sop.*, offers an alternative method of multiplication [3]. It is generally propagated by vegetative methods like cutting, layering, budding and grafting [4]. Although propagation by vegetative means is a predominant technique in roses, yet it does not ensure healthy and disease-free plants. Moreover, dependence on season and slow multiplication rates are some of the other major limiting factors in conventional propagation. In the last few years, *in vitro* propagation has revolutionized commercial nursery business [5]. Significant features of *in vitro* propagation procedure are its enormous multiplicative capacity in a relatively short span of time; production of healthy and disease free plants; and its ability to generate propagules around the year [6, 7].

Several micropropagation protocols have been proposed in *Rosa hybrida* L. however, only cost efficient protocol capable of producing a larg number of good quality micro-shoots in several subculture can be successful [8]. The addition of suitable hormones can induce root growth and then the plants be placed in soil and grown in the normal manner [1, 2]. Today, rose tissue culture are exploited for various purposes as, basic anatomical and physiological research, creation of androgenetic haploids, micro propagation from calli, immature embryos and or protoplast [2]. So far no report has been published for rose micropropagation in Iran. The aim of this study is to introduce a suitable and reproducible protocol for micropropagation of *Rosa hybrida* cultivar Black Red under *in vitro* condition.

MATERIALS AND METHODS

In this study a commercial *Rosa hybrida* cultivar Black Red was used for *in vitro* culture. Healthy young plants were grown in the glasshouse under normal day

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Table 1: Combination of MS medium

Media name	Combination
T1	0.1 mg l^{-1} IBA + 30 g l^{-1} sucrose
T2	1.0 mg l^{-1} NAA + 30 g l^{-1} sucrose
T3	0.5 mg l^{-1} BA + 30 g l^{-1} sucrose
T4	5.0 mg l^{-1} BA + 30 g l^{-1} sucrose
T5	0.1 mg l^{-1} IBA 5mg l^{-1} BA + 40 g l^{-1} sucrose
T6	40.0 g l^{-1} sucrose
T7	0.1 mg l^{-1} NAA + 5mg l^{-1} TDZ + 40 g l^{-1} sucrose
T8	1.0 mg l^{-1} NAA + 5mg l^{-1} BA + 30 g l^{-1} sucrose
T9	0.1 mg l^{-1} NAA + 0.5mg l^{-1} 2iP + 30 g l^{-1} sucrose
T10	0.5 mg l^{-1} NAA + 0.5mg l^{-1} BAP + 30 g l^{-1} sucrose

light for 2-3 months. As explant, young internodes having at least one axillary bud were harvested from fully grown plants. Explants were then washed with tap water and transferred to 70% ethanol for 1-1.5 min and then were surface sterilized with sodium hypochlorite (2%v/v) containing two drops of Tween 80 for 20 min followed by 3-4 washes with sterile water. Explants were then cultured on MS medium [9] supplemented with sucrose (30-40 g l^{-1}), agar (8 g l^{-1}) and combination of different hormones according to Table 1. The pH of media was adjusted to 5.8 then were autoclaved for 20min at 121°C.

All culture treatments with 8 replications were incubated in the culture room at 25°C±2 and photoperiod of 16 h light and 8 h dark cycle. After 4, 8 and 12 weeks, growth parameters such as stem length, root length, number of explants showing necrosis and chlorosis were evaluated. All data were analysed using SPSS program with Tukey test. In some cases nonparametric tests have been applied.

RESULTS

Effects of different combination of hormones and sucrose in the medium on shoot and root formation from nodal culture of rose are presented in Table 2. Within 4 to 12 weeks the most responsive medium for shooting were medium T3, T5, T6, T7 and T10. The lowest shoot multiplication was observed on medium T2 while the highest shoots were formed on medium T5. After 4-12 weeks, T7, T2 and T10 medium showed the highest rooting on explants however, rooting on T1 medium was the lowest. The number of explants showed necrosis and chlorosis was the lowest on medium T3 and T5. After acclimatization, rooted plants were transferred from tissue culture containers to the glasshouse, under normal daylight, Aprox. 70% of plants were able to continue to grow and survived. The overall results of

Table 2: Comparison of effect of different media on nodal culture of rose (RL: root length, SL: shoot length, C: number of chloresis, N: number of necrosis) after 12 weeks. In each column data are mean value (±std) and similar letters are not significant according to Tukey test ($p<0.05$)

Media		C	N	RL (cm)	Media No.
T1	Mean	0.875b	2.000b	1.500b	2.000d
	std	0.835	1.069	0.535	0.378
T2	Mean	0.1000c	3.000a	3.000a	1.000e
	std	0.000	0.000	0.845	0.378
T3	Mean	0.000	0.000	0.000	3.000b
	std	0.000	0.000	0.000	0.655
T4	Mean	3.000a	1.000c	0.000	2.000d
	std	0.756	0.756	0.000	0.378
T5	Mean	0.000	0.000	0.500c	4.500a
	std	0.000	0.000	0.463	0.535
T6	Mean	1.000c	1.000c	0.000	3.500b
	std	0.756	0.756	0.000	0.655
T7	Mean	1.000c	1.000c	0.000	3.500b
	std	0.756	0.756	0.000	0.655
T8	Mean	0.000	0.000	0.000	2.503d
	std	0.000	0.000	0.000	0.752
T9	Mean	1.000c	1.000c	0.000	2.500d
	std	0.756	0.535	0.000	0.756
T10	Mean	1.000c	1.000c	2.000b	3.500b
	std	0.756	0.756	0.378	0.463

Fig. 1: *In vitro* propagation steps of *Rosa hybrida* cultivar Black Red. A: shoot proliferation, B: root formation, C: flower formation, D: growing plant after acclimatization

shoot and root formation as well as flower setting are illustrated in Fig. 1.

DISCUSSION

Tissue culture techniques are used extensively for growing plants as commercial and scientific researches. This process involves growth of new plants from small pieces of plant tissue in a nutrient medium in sterile conditions. Under suitable conditions, plants can be induced to rapidly produce new shoots and these can be subdivided to produce more plants [1]. In this study, according to the responses of explants cultured on different medium, T5 medium was relatively the best and the most favorite treatment for shoot and root formation. Increasing of sucrose as a carbon sources for photosynthesize in the medium affects on shoot initiation and shoot growth while, auxin generally decrease shoot proliferation [8]. Rooting was also affected by MS salt concentration and low salt and IAA supplementation resulted in better root formation. Hyndman *et al.* [10], recommended decreasing medium salt concentration from MS medium generally increase rooting for rose propagation. In contrast, we found that full strength of MS medium is able to initiate root formation. The highest number of roots was observed in explants cultured on T2 medium. When chlorosis and necrosis were measured within 4-12 weeks, chlorosis in 4th week was very low in T1, T5 and T10 but at the same time the most chlorosis occurred in medium T7 and T8. After 8 weeks chlorosis was almost observed in all treatments. However, as a general pattern in *in vitro* rose multiplication, when explants remaine in the medium for longer time, there is a higher risk of chlorosis as well as necrosis. This phenomenon might be as a result of phenolic compounds leaking from explants "especially woody plants" to the medium and their subsequent oxidation produce toxic componds Alternative reason can be accumulation of ethylene in the culture vessels as a result of low gas exchange. Chlorosis and necrosis were also influenced by auxin level [2, 10-12], for example, the highest number of chlorosis and necrosis was observed in medium T4. The highest shoot proliferation occurred in full strength MS medium supplemented with T5 phytohormones however, the most appropriate medium for simultaneous proliferation of shoot and root was T10 medium. It has been reported that, in *Rosa. Persica+R. Xanthina*, shoot regeneration from callus culture require BA and NAA but, the cytokinin is required at about 20 times the concentration of the auxin [2, 10]. However, some micro propagation media contain gibberlin, but it can reduce shoot survival and leaf expansion. Although proliferation of rose shoot is relatively easy but rooting is frequently difficult [13].

A successful micropropagation protocol proceeds through a series of stages, each with a specific set of requirements. These are (i) initiation of aseptic cultures, (ii) shoot multiplication, (iii) rooting of microshoots and (iv) hardening and field transfer of tissue culture raised plants the rate of shoot multiplication depending upon position of node on the stem in different cultivars of *R. hybrida*. The buds nearest to the apex and closest to the base of the stem exhibited the slowest rate of development, but those from the mid-stem region grew very rapidly. The potential of axillary bud outgrowth, which is related to position on the main axis, appears to be determined by a balance among several hormones [14, 15]. Further, the axillary buds in the mid-region of plants have the potential to grow; however, they cannot grow on intact plants due to apical dominance. Removing apical dominance could change this balance. The finding of Rout *et al.* [16] and Bressan *et al.* [8] on the differences in the rate of shoot multiplication depending upon position of node on the stem could be understood in this context. Marcelis van Ackerand Scholten [13] in *R. hybrida*. Salehi and Khosh-Khui [17] found that the explant length and diameter played significant roles in proliferation and shoot growth of miniature roses. They also reported that the best rates of shoot growth and proliferation were obtained in explants with a length of about 9.0-10.0 mm and diameter of about 3.0-3.5 mm, we have found more and less the same observation in this study (data are now shown). However, no such a correlation was observed in *Rosa damascena* and *R. bourboniana* reported by Pati, [18]. Alekhnoand Vysotskii [19], while working with Hybrid Tea, Floribunda and miniature rose cultivars, reported that growing shoots in horizontal position during proliferation almost doubled the axillary branching as compared with growing shoots in vertical position. The increased axillary shoot proliferation by placing the explant in horizontal position could be attributed to greater uptake of the medium constituents due to increased contact with the medium [20]. Similar results were also reported in pears [21] and liliac [22].

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