American-Eurasian J. Agric. & Environ. Sci., 10 (6): 962-967, 2011
ISSN 1818-6769

Direct Shoot Regeneration on Three Cultivars of *Rosa hybrida* Using Five Explant Types and Different Hormone Concentrations

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Abstract: The regeneration potential of different rose genotypes and five explants types were compared in order to use the results in further *in vitro* breeding. Experiments on direct shoot regeneration were carried out on three *in vitro* cultivars of *Rosa hybrida* cv 'Apollo', 'Dolce Vita' and 'Maroussia'. Four types of leaf segments (complete leaf with its subtending petiole, leaflet and its subtending petiole, petiole without leaflets and leaflet without petiole) and internodes (0.2 to 0.4 cm) were cultured in ½ MS media containing different concentrations of thidiazuron (0, 7 and 14 μM) in combination with different concentrations of á- naphthalene acetic acid (0, 0.5 and 1 μM). The present investigation was the first report on direct shoot regeneration in the studied cultivars. The results showed that the highest percentages of regeneration (67%) and (31%) for cv 'Apollo' and 'Dolce Vita' were observed in media containing 14 μM TDZ and 7 μM TDZ respectively when using the internodes. Whereas for the cv 'Maroussia' the highest percentage of regeneration (75%) was observed in the medium containing 14 μM TDZ + 1 μM NAA when using the leaflet without petiole. The results indicated that regeneration was highly related to the type of explant and genotype used and there was a significant correlation between percentage of regeneration and number of adventitious shoots per explants. Present study also was the first report on obtaining relatively high percentage of direct regeneration from internodal explants of *Rosa hybrida*. Regardless of the type of explant and media composition the highest and lowest percentages of regeneration were observed in the cv 'Maroussia' and 'Dolce Vita' respectively. Regardless of the type of explant and genotype, the best regeneration was obtained in the medium containing 14 μM TDZ and 1 μM NAA.

Key words: Cultivars · Direct regeneration · Internodes · Leaf segments · *Rosa hybrida* · TDZ

INTRODUCTION

Roses (*Rosa hybrida*) are one of the most important ornamental plants in the world which are highly used in commercial flower industry. They are usually propagated by traditional methods, but *in vitro* regeneration systems are required for rose breeding using modern biotechnology techniques. The availability of an efficient regeneration system is also useful for rapid propagation of elite rose individuals. Since better penetration of chemical mutagens occurs in small and thin explants such as leaves and internodes compared to the more complex tissues such as meristems, therefore, a regeneration protocol for these explants is highly valuable. The regeneration protocol is also beneficial in agro bacterium mediated transformation and direct gene transformation in leaf discs. Regeneration of adventitious shoots in roses has been reported from immature zygotic embryos [1] and from *in vitro* and *in vivo* grown leaves and shoot explants [2, 3]. TDZ a cytokin in like compound is found to be very effective for stimulation of adventitious shoots in Rosaceae. Dubois and De Vries, (1995) reported a two step regeneration method by directly including adventitious buds from *in vivo* leaflets of roses [3]. Based

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on this method, Ibrahim and Debergh, (2001) made some modification and developed the method for *in vitro* leaflet regeneration [4]. According to their report direct organogenesis was rapid, convenient and highly efficient; however, it has not been applied in many rose cultivars. The aim of the present study was to optimize direct regeneration potential of five explant type in three different rose cultivars in order to use the results in further *in vitro* breeding studies.

MATERIALS AND METHODS

Plant Material and General Procedures: Three commercial rose genotypes; *Rosa hybrida* cv 'Apollo', 'Dolce Vita' and 'Maroussia' were supplied by the Rose Germplasm Collection at Agricultural Biotechnology Research Institute of Iran (ABRII). They were sterilized according to our previous report [5] and were transferred to induction medium containing full strength MS [6] basal medium (without hormone). Nodal sections were cultured on elongation medium containing VS medium [7] with 1 μM BAP (6- benzene amino purine), 3% sucrose, 0.7% plant agar (Duchefa™). The *in vitro* leaves and internodes were used in induction of direct regeneration experiments. The pH of all media was adjusted to 5.8 using 1.0 N potassium hydroxide (KOH) or 1.0 N hydrochloric acid (HCl) before adding 7 gl^{-1} plant agar. Media were autoclaved for 20 min at 121°C and 1.2 $kg.cm^2$ pressure. All cultures were placed under high pressure metal halide lamps (PPFD 60 $\mu mol.m^{-2}.s^{-1}$ at the plant surface) on a 16/8 hour light/dark cycle in a culture room maintained at 20±2°C.

Direct Shoot Regeneration: The *in vitro* shoots were cultured on elongation medium for four weeks. The leaf and internode explants were detached from the midpoint of the 4-5 cm long shoots. Five types of explants including complete leaf with its subtending petiole (number 1), a leaflet and its subtending petiole (number 2), petiole without leaflets (number 3), leaflets without petiole (number 4) and 0.2 to 0.4 cm internodes (number 5). The explants were treated in media containing half- strength MS medium, full vitamins, 100 $mg.l^{-1}$ Casein hydrosylate and 60 μM silver nitrates supplemented with three levels of TDZ (0, 7 and 14μM) in combination with three levels of NAA (0, 0.5 and 1 μM). Because TDZ and silver nitrate are sensible to heat they were filtered through a Milipore filter (0.22 μm pore size) and added to the autoclaved media. Explants were placed in the petri dishes with their abaxial side touching the media. The cultures were kept at the first induction stage, in the dark for 7 days and then were transferred to the second induction stage, in the red light (15 $\mu molm^{-2}s^{-1}$), using red plastic films on petri dishes for 7 more days. Finally the cultures were placed in light conditions under high pressure metal halide lamps (60 $\mu mol.m^{-2}.s^{-1}$) for 14 days.

The explants were then transferred to the regeneration medium containing MS culture medium, 2.2 μM BAP, 0.3 μM GA3 and 0.05 μM NAA. Percentage of regenerated shoots and the number of shoots per explant were recorded at the end of regeneration stage (Week eight).

Experimental Design and Statistical Analysis: Each treatment was repeated five times and each repeat had four replicates. The experimental design was a factorial randomized-block arrangement. Data were analyzed using statistical programs MSTAT-C and SPSS. Statistically significant averages were compared using Duncan's Multiple Range tests. Graphs were plotted with the Excel program.

RESULTS

Interactive Effect of Explants Type, Hormone Concentration and Genotype on Direct Shoot Regeneration: There was a significant difference between different explant type, genotype and culture media in the percentage of regeneration and number of shoots per explant. The highest percentage of regeneration (75%) and maximum number of shoots per explant (4.4) were observed in cv 'Maroussia' with explant 4 (leaflets without petiole) and explant 2 (leaflet with its subtending petiole) on media containing 14μM TDZ+1 μM NAA and 7μM TDZ+1μM NAA respectively (Table 1 & 2). In cv 'Apollo' the highest percentage of regeneration (67%) and highest number of shoots per explant (2.6) were detected in explant 5 (internode) on media containing 14μM TDZ+0μM NAA and 7μM TDZ+0.5μM NAA respectively (Table 1 & 2). The lowest percentage of regeneration was observed in the cv 'Dolce Vita' where the maximum percentage of regeneration (31%) and maximum number of shoots per explant (0.9) was observed with explant 5 (internode) on medium including 7μM TDZ+0μM NAA (Table 1 & 2).

Dubois *et al*., (2000) reported a significant correlation between the percentage of regeneration and number of shoots per explant from *in vivo* leaves of roses [8]. They explained that if regeneration capacity depends on sensitivity of tissue to cytokenins, therefore, as

Table 1: Percentage of direct regeneration in five explant type and three genotypes using different concentrations of NAA and TDZ (The numbers have been rounded)

Percentage of direct regeneration										
		0µM NAA			0.5µM NAA			1µM NAA		
Cultivar	Explant type	0µM TDZ	7µM TDZ	14µM TDZ	0µM TDZ	7µM TDZ	14µM TDZ	0µM TDZ	7µM TDZ	14µM TDZ
''Apollo''	1	0	16t	20s	0	30p	30p	0	50i	35m
	2	0	11u	21rs	0	35m	21rs	0	26q	40l
	3	0	7v	25q	0	11u	11u	0	16t	30op
	4	0	0	12u	0	7v	7v	0	21rs	35m
	5	0	58g	67d	0	25q	50i	0	46j	55h
'Dolce Vita'	1	0	7v	7v	0	21r	0	0	0	7v
	2	0	7v	7v	0	7v	0	0	0	0
	3	0	7v	7v	0	0	0	0	0	0
	4	0	11u	0w	0	0	0	0	7v	0
	5	0	31o	11u	0	12u	7v	0	12u	7v
'Maroussia'	1	0	16t	40l	0	60f	55h	0	65e	55h
	2	0	33n	20s	0	55h	50 i	0	71b	70c
	3	0	7v	40l	0	31op	30 op	0	40l	40l
	4	0	30op	7v	0	25q	45 k	0	30p	75a
	5	0	30op	16t	0	40l	31 o	0	30op	16t

Table 2: Number of regenerated shoots in five explant types and three genotypes using different concentrations of NAA and TDZ (The numbers have been rounded)

Number of regenerated shoot per explant										
		0µM NAA			0.5µM NAA			1µM NAA		
Cultivar	Explant type	0µM TDZ	7µM TDZ	14µM TDZ	0µM TDZ	7µM TDZ	14µM TDZ	0µM TDZ	7µM TDZ	14µM TDZ
''Apollo''	1	0	0.6s	2hi	0	1.0pq	1.8j	0	2.2g	1.9ij
	2	0	0.6s	1.8j	0	1.8j	0.8r	0	0.9qr	1.5kl
	3	0	1.0pq	1.0pq	0	0.8r	0.4t	0	1.2no	1.5kl
	4	0	0	0.2 u	0	0.2u	0.6s	0	0.6s	1.5kl
	5	0	1.9ij	1.9 hij	0	2.6e	1.9hij	0	1.3mn	2.1gh
'Dolce Vita'	1	0	0.2u	0.2 u	0	0.4t	0	0	0	0.4t
	2	0	0.2u	0.4 t	0	0.2u	0	0	0	0
	3	0	0.2u	0.2 u	0	0	0	0	0	0
	4	0	0.6s	0	0	0	0	0	0.4t	0
	5	0	0.9qr	0.4 t	0	0.4t	0.4t	0	0.4t	0.2u
'Maroussia'	1	0	0.4t	2 hi	0	1.8j	2.7e	0	2.5e	2. 6e
	2	0	3.2c	3.3bc	0	3.4b	2.9d	0	4.4a	2.3f
	3	0	0.2u	1.4lm	0	1.6k	1.6k	0	1.06op	2.0hi
	4	0	0.8r	0.4t	0	1.4lm	3.3b	0	1.2no	1.5kl
	5	0	1.2no	1.2no	0	1.5kl	1.6k	0	1.3mn	1.8j

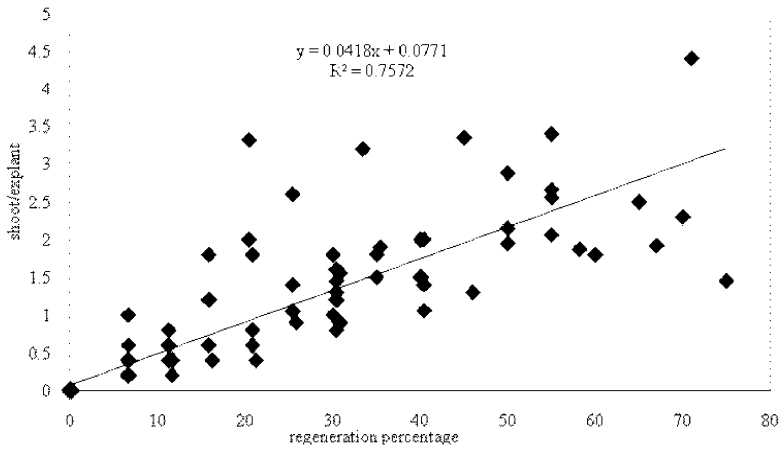

Fig. 1: Correlations between percentage of regeneration and number of adventitious shoots per explants. (n=135. 3cvs*9 media*5 explant)

sensitivity of genotypes increases more explants would form more adventitious buds in a shorter time. Our results also indicated that there was a significant correlation between the percentage of regeneration and number of shoots per explant (Figure 1). In all of the treatments (different explant types, genotypes and culture media) as the percentage of regeneration increased the number of shoots per explant was also increased (Table 1). This could be attributed to the fact that different explant types and genotypes have different sensitivity towards the hormone combinations and as the sensitivity increases the percentage of regeneration and number of regenerated shoot per explant also increases. For example in the cv 'Maroussia' the highest percentage of regeneration and maximum number of shoots per explants were recorded on the media containing TDZ and NAA. Whereas in the cv 'Dolce Vita' and 'Apollo' the highest percentage of regeneration and maximum number of shoots per explants were obtained on medium containing TDZ without NAA, which is probably due to the presence of high endogenous levels of NAA in them.

Effect of Explant Type on Direct Shoot Regeneration: There was a significant difference between the explants in their response to direct regeneration (Tables 1 & 2). The highest percentage of regeneration in the cv 'Maroussia' was observed in the explant 2 (leaflet and its subtending petiole), whereas the highest percentage of regeneration in the cv ''Apollo'' and 'Dolce Vita' was obtained on the explant 5 (internode) (Table 1). Other reports have also stated that the success of *in- vitro* shoot formation may depend on the type of explant used [9, 10, 11, 12]. In the present study the lowest percentage of regeneration was observed in the explants 3 & 4 (petiole without the leaflet & leaflet without the petiole), may be because these explants were smaller than the two other leaf explants and had less carbohydrates or endogenous growth hormone contents to produce shoots. This was supported by Gao and Bao, (2005) who also reported low regeneration from petiole explants (10%), but 56% regeneration from leaflet explant of *Rosa hybrida* cv Samantha [13]. Matand and Prakash, (2007) reported that younger leaves in peanut are more responsive than the mature ones and the whole leaf and lamina were one of the most organogenetically responsive explant types, while the petiole was the least responsive [14].

This is the first report obtaining direct buds from internodal explants of *Rosa hybrida*. Nodal explants of three studied cultivars ''Apollo'' (67%), 'Dolce Vita' (31%) and 'Maroussia' (40%) had relatively high percentage of regeneration (Table 1). Belaizi *et al*., (1991) obtained 12.6% regeneration from internodal segments of apple cv Golden delicious[15] whereas Wang *et al*., (2009) achieved 100% efficiency rate in peppermint (*Mentha x piperita*) using 5- to 7-mm-long second internode stem segments of three week old stock plants[16]. Cirak *et al*., (2007) also found that internodal explants of *Hypericum bupleuroides* were more responsive than leaf tissues to direct and indirect plant regeneration [17]. In the cv 'Apollo' and 'Dolce Vita' the highest percentage of regeneration (67%) and (31%) respectively were observed from the internodal explants. In the media containing 7 and 14µM TDZ without NAA nodal explants produced adventitious shoots on both ends of the explant.

The highest percentage of regeneration was detected in explant 1 & 2 (leaf & leaflet with subtending petiole) in the cv 'Maroussia' which could be due to the fact that the cells at the petioles of young leaf & leaflet may stay in primary and dividing condition and they are more sensitive to plant growth regulators and therefore are easier to differentiate into organized structures.

Effects of Different Hormone Combinations on Direct Shoot Regeneration: In the media without any hormone (control) there was no regeneration and media containing only NAA the explants produced only roots. Regardless of the type of explant and genotype, the best regeneration was obtained in the medium containing 14 µM TDZ and 1 µM NAA. It was previously reported that 6.8 µM TDZ alone is the best concentration for direct regeneration of *Rosa hybrida* cultivars, [4, 8]. But in the present investigation increasing NAA concentration to 1 µM increased the percentage of regeneration significantly. These results is according to Uranbey (2005) that TDZ induced more explants producing shoots and higher mean number of shoots per explant as compared to BAP at the same concentrations in *Hyoscyamus niger* [19]. In contrary Tang *et al*., 2002 found that BAP was more effective than TDZ in regenerating shoots of sour cherry leaves explants [20].

Response of Different Cultivars to Direct Shoot Regeneration: In the present investigation significant differences between different genotypes in their response to adventitious shoot regeneration and number of shoots per explant was observed (Tables 1 & 2). The highest and lowest percentages of regeneration were observed in the cv 'Maroussia' and 'Dolce Vita' respectively Significant genotype differences in adventitious shoot regeneration were also reported by Pavingerova, 2009 in different

rhododendron genotypes [20]. However, Dubois *et al*., (2000) reported that *in vivo* rose's cultivars are not very recalcitrant to direct shoot regeneration. They reported 63 to 100% shoot regeneration and 0.5 to 4.0 shoot number per leaf explant among 24 glasshouse grown rose cultivars.

CONCLUSION

- Present study for the first time reported direct adventitious shoot regeneration from the three studied cultivars; 'Maroussia', 'Apollo' and 'Dolce Vita'. The highest percentage of regeneration of cv 'Maroussia' (75%) was observed in the medium containing 14 μM TDZ + 1 μM NAA when using the explant 4 (leaflet without petiole). Whereas the highest percentages of regeneration in cultivars ''Apollo'' (67%) and 'Dolce Vita' (31%) were observed in media containing 14 μM TDZ and 7 μM TDZ respectively, using the explant 5 (internodes).
- Genotype, explant type and medium composition are considered the three main factors affecting *in vitro* plant regeneration in many plant species. Our results indicated that in all of the treatments explant types, genotypes and culture media affected the percentage of regeneration and number of shoots per explant.
- There was a significant correlation between the percentage of regeneration and number of shoots per explant; as the percentage of regeneration increased the number of shoots per explant was also increased. This was attributed to the fact that different explant types and genotypes have different sensitivity towards the hormone combinations and as the sensitivity increases the percentage of regeneration and number of regenerated shoots per explant also increases.
- Regardless of the type of explant and media composition the highest and lowest percentages of regeneration were observed in the cv 'Maroussia' and 'Dolce Vita' respectively.
- Regardless of the type of explant and genotype, the best regeneration was obtained in the medium containing 14 μM TDZ and 1 μM NAA.

ACKNOWLEGMENT

This work was a part of PhD thesis on "Mutation breeding of some *Rosa hybrida* cvs for investigation morphological changes using chemical mutagens" Science and Research Branch, Islamic Azad University, funded by Agricultural Biotechnology Research Institute of Iran (ABRII) (Project number: 12-05-05-01-8704-88003).

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