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Essential factors for *in vitro* regeneration of rose and a protocol for plant regeneration from leaves

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Abstract

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In vitro propagation of *Rosa hybrida*, L. cv. 'Eiffel Tower' was improved by the addition of thidiazuron (TDZ) and silver nitrate ($AgNo_3$) to the culture medium. The combination of auxin and cytokinins was indispensable for inducing response from leaf discs. Maintaining cultures under dark was better than light for callus formation and quality. The source of explants was vital in the regeneration process where*in situ* explants produced callus while, *in vitro* explants regenerated somatic embryos and shoots. Gibberellic acid (GA_3) had a favorable effect where *in vitro* explants showed somatic embryogenesis with no shoots on media containing TDZ however, 37% of explants regenerated shoots directly on medium containing GA_3. The presence of benzyl adenine (BA) was essential for shoot elongation, and indole butyric acid (IBA) was better than indole acetic acid (IAA) for rooting. The optimum conditions produced rooted plants from leaf discs within ten weeks. The reported results clarify factors controlling *in vitro* regeneration of *R. hybrida*, and provide a rapid protocol allowing further improvements of rose.

Keywords: *Rosa hybrida*; leaf discs; embryogenesis; organogenesis; direct regeneration

Rose is the most economically important flowering plant cultivated in the world. It includes more than 200 species which are used for inside and outside decoration, cut flowers, essential oils as well as medicinal and aromatic purposes. *Rosa hybrida* is the most economically important species which includes most commercial cultivars. 'Eiffel Tower' is a scented rose cultivar produced commercially in Egypt and other countries for its cut flowers and essential oil.

Conventional breeding of rose is limited by its polyploid and highly heterozygous nature (Kim et al. 2004), long and slow propagation (Pati et al. 2006). It is also affected by climatic changes and do not ensure healthy or uniform plants. Biotechnology provides a tool capable of overcoming some of these restrictions. *In vitro* culture can offer a great potential for rapid mass production of cultivars, the generation of somaclonal variants and the insertion of interesting genes via transformation technology. The establishment of an efficient and reliable protocol for plant regeneration is an important prerequisite step needed for the successful implementation of biotechnological techniques for plant improvement.

Many trials were conducted for *in vitro* regeneration of different rose species. *R. damascena* was regenerated indirectly from callus derived from stem tissue (Ishioka, Tanimoto 1990), or directly via leaf explants of *in vitro*-raised shoots (Pati et al. 2004). Direct regeneration was also reported on *R. canina* (Moallem et al. 2012). For *R. hybrida*, shoot regeneration has been reported from immature embryos (Burger et al. 1990), callus derived from *in vitro* and *in vivo* leaves and shoots (Rout et

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al. 1992) and embryogenic cell-derived protoplasts (Kim et al. 2003). In plants regenerated from various vegetative embryogenic tissues of Meirutral cultivar, somaclonal variants were found (Arene et al. 1993). *In vitro* leaf explants were used for plant regeneration in a two-stage procedure by the incubation of leaf discs on induction medium before transferring to regeneration medium (Ibrahim, Debergh 2001). Somatic embryogensis and plant regeneration were also obtained using *in vitro* leaf explants after incubation on induction medium followed by several subcultures (Kim et al. 2004).

Few reports deal with direct regeneration from *R. hybrida*. In previous studies, we found *in vitro* leaves efficient for direct and indirect regeneration of pelargoniums, but somaclonal variants were resulted from callus formation and the long regeneration procedure (Hassanein, Dorion 2005, 2006). A rapid regeneration system is needed for its efficient use in a plant improvement program. Determination of factors controlling regeneration procedure can also help in further improvement and application on other species. To the best of our knowledge, there is no report on plant regeneration of the commercially important scented hybrid tea rose (*R. hybrida* cv. 'Eiffel Tower'). We previously reported an efficient protocol for the *in vitro* propagation of that cultivar (Mahmoud et al. 2011). Here, we report on the factors controlling its direct or indirect regeneration, and a protocol for rapid plant regeneration allowing mass production and further improvement using the transformation approach.

MATERIALS AND METHODS

Production of in vitro mother plants as source of explants. Mother plants of hybrid tea rose, *R. hybrid* cv. 'Eiffel Tower', were introduced under *in vitro* conditions following our previously reported propagation protocol (Mahmoud et al. 2011). Nodal segments of 1 cm length were taken from two-year old *in situ* grown plants. Explants were sterilized using 20% sodium hypochlorite solution, then cultured on MS (Murashige, Skoog 1962) modified medium containing half strength macronutrients, micronutrients, vitamins, Fe EDTA, 20 mg/l sucrose, 1 mg/l indole acetic acid (IAA), 9 g/l bacto agar and 2 g/l active charcoal. To improve the protocol, the addition of thidiazuron (TDZ) at 0.5 mg/l and/or silver nitrate ($AgNo_3$) at 10 mg/l to culture medium was investigated (Akasaka-Kennedy et al. 2005). Five explants were cultured per Petri dish (95 mm) containing 25 ml of medium. Each treatment consisted of nine dishes in three replicates, and the experiment was repeated twice. Cultures were maintained in the growth chamber under 16 hours daily light (70 μmol/s/m^2), 25 ± 1°C and 60% humidity.

Explant preparation and culture medium. Leaf explants of *R. hybrid* cv. 'Eiffel Tower' were obtained from *in vitro* mother plants, one month after subculture, or two-year old plants grown in the greenhouse (*in situ*). Young fully developed leaves of *in situ* grown plants were washed under tap water then surface sterilized using 70% ethanol before transferring to 15% sodium hypochlorite solution containing drops of Tween-80 for 20 min and finally rinsed three times in sterile distilled water. Leaves from both sources were cut into leaf discs of 0.5 to 0.75 cm diameter, then placed with abaxial side down, in Petri dishes of 95 mm diameter containing 25 ml medium, 7–8 leaf discs per dish. The basal medium(BM) contained MS macronutrients at half strength, MS micronutrients, vitamins of Morel, Wetmore (1951), Fe EDTA, 20 g/l sucrose, 10 mg/l $AgNo_3$and 9 g/l bacto agar. The pH was adjusted to 5.8 and autoclaving was achieved at 113°C for 20 minutes.

Shoot regeneration from leaf discs and studied factors. For plant regeneration from leaf discs, many factors were studied. The type and concentration of plant growth regulators (PGR) were investigated in a factorial experiment including three PGRs and four concentrations. The PGRs included naphthalene acetic acid (NAA), benzyl adenine (BA) and thidiazuron (TDZ) at concentrations of 0, 0.5, 1 and 2 mg/l. The leaf discs were obtained from *in situ* grown plants and prepared as mentioned above then cultured in Petri dishes containing the basal medium (BM) and one of the treatments. Cultures were maintained in the growth chamber under dark conditions for a week then exposed to a 16 h photoperiod. The daily temperature and humidity were 25 ± 1°C and 60%, respectively.

The combination among the three PGR (NAA, BA and TDZ) at three concentrations (0.5, 1 and 2 mg/l) were studied under two different culture conditions (dark and light). After preparation and sterilization as described above, leaf discs of *in situ* grown plants were cultured in Petri dishes containing the BM medium and one of twenty seven NAA + BA + TDZ combinations. Cultures were either

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covered to create dark conditions, or exposed to 16 h of light (70 µmol/s/m^2) in a growth chamber of 25 ± 1°C and 60% humidity.

The source of explants was also studied under the best PGR combinations as determined in the previous experiment. Leaf discs were taken from *in vitro* grown plants or *in situ* grown plants after disinfection, and prepared as previously described. Explants were cultivated on BM medium containing one of six PGR combinations including NAA + BA in combination with thidiazuron (TDZ) or gibberellic acid (GA_3) at 0.5, 1 and 2 mg/l, to study the effect of GA_3 on plant regeneration. Cultures were kept in the dark under 25 ± 1°C and 60% humidity.

All the experiments performed in three replicates and repeated twice.

Shoots elongation and plantlets rooting. After four weeks of culture, growing buds or regenerating shoots were individually transferred to jars containing 25 ml of fresh basal medium (BM) containing BA at 0, 0.5 and 1 mg/l in combination with IBA or IAA at 0, 0.5 and 1 mg/l for shoot elongation. Twenty explants in four replicates were cultured per treatment. Cultures were kept in the growth chamber with 16 h photoperiod from white fluorescent light, 25 ± 1°C and 60% humidity.

For rooting, elongated shoots with 3–4 nodes were individually transferred to jars containing 25 ml of fresh basal medium (BM) supplemented with IAA or IBA at 0, 0.5, 1 or 2 mg/l. Sixteen plantlets in four replicates were tested per treatment. Cultures were maintained under similar elongation conditions.

Collection of data and statistical analysis. For all experiments, data were collected four weeks after culture. The percentages of green or grown buds and the percent of buds showing phenolic compounds (phenols %) were calculated related to the total cultivated explants (Mohmoud et al. 2011). The mean of shoot length was measured for growing buds. The percentage of leaf discs showing callus compared to the total cultivated leaf discs was recorded. The response percentage was calculated as the percent of leaf discs showing callus formation or embryogenesis related to the initial cultivated number of leaf discs. Shoot regeneration (%) was similarly recorded as the percent of explants regenerating shoots directly. The mean of shoot length and number of leaves per shoot were determined per elongated shoot. Rooting percentage was recorded as the percent of rooting plantlets related to the cultivated ones. Data were subjected to analysis of variance (ANOVA) to determine significant differences, and the least significant difference at 0.05 were used for the comparison of means using SAS 9.1.3 program. For certain results, means were shown with the confidence interval at significance level of 0.05 using Excel 2010 program.

RESULTS AND DISCUSSION

Production of in vitro mother plants

The production of mother plants under *in vitro* conditions using nodal segments showed significantly higher grown buds on media containing thidiazuron or silver nitrate compared to control (Table 1). The highest rate of bud growth (100%) was obtained with medium including both compounds where all buds developed shoots of good length. It was also noticed that media containing TDZ have no phenolic compounds compared to other tested media. These obtained results proved the possibility of improving our previously reported protocol (Mahmoud et al. 2011) by the addition of thidiazuron(TDZ) and silver nitrate (SV). The promoting effect of TDZ and SV on shoot proliferation was reported on other plant species (Geetha et al. 2016). The beneficial effect of SV may be related to its inhibition of ethylene action (Ozudogru et al.

Table 1. Propagation of *in vitro* mother plants from nodal segments of *R. hybrid* cv. 'Eiffel Tower' as affected by thidiazuron (TDZ) and silver nitrate ($AgNo_3$), four weeks after culture

Treatments	Green buds (%)	Grown buds (%)	Phenols (%)	Shoot length (cm)
BM (control)	100.0	88.9 ± 0.07	0.7 ± 0.15	2.3 ± 1.04
BM + 0.5 mg/l TDZ	100.0	93.3 ± 0.05	0.0	2.0 ± 0.61
BM + 10.0 mg/l $AgNO_3$	100.0	93.3 ± 0.05	0.3 ± 0.10	2.7 ± 0.77
BM + 0.5 DZ + 10 mg/l $AgNo_3$	100.0	100.0	0.0	3.2 ± 0.96

BM – basal medium containing (mg/l) indole acetic acid (IAA); means of two repetitions with confidence interval at 0.05

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Table 2. Effect of type and concentration of plant growth regulators on callus formation (%) from leaf discs of *R. hybrid* cv. "Eiffel Tower"

NAA (mg/l)	BA (mg/l)	TDZ (mg/l)				Mean (NAA × BA)	Mean (NAA)
		0	0.5	1	2		
0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	0.5	0.0	0.0	0.0	0.0	0.0	
	1.0	0.0	0.0	0.0	0.0	0.0	
	2.0	0.0	0.0	0.0	0.0	0.0	
Mean (NAA × TDZ)		0.0	0.0	0.0	0.0		
0.5	0.0	0.0	85.7	96.4	85.7	67.0	85.9
	0.5	78.6	82.1	92.9	92.9	86.6	
	1.0	89.3	89.3	96.4	100.0	93.8	
	2.0	96.4	96.4	96.4	96.4	96.4	
Mean (NAA × TDZ)		66.1	88.4	95.5	93.8		
1.0	0.0	0.0	89.3	92.9	92.9	68.8	87.5
	0.5	89.3	89.3	89.3	92.9	90.2	
	1.0	100.0	92.9	96.4	96.4	96.4	
	2.0	89.3	89.3	100.0	100.0	94.7	
Mean (NAA × TDZ)		69.7	90.2	94.7	95.6		
2.0	0.0	60.7	92.9	92.9	92.9	84.9	94.0
	0.5	92.9	92.9	96.4	96.4	94.7	
	1.0	96.4	96.4	100.0	100.0	98.2	
	2.0	96.4	96.4	100.0	100.0	98.2	
Mean (NAA × TDZ)		86.6	94.7	97.3	97.3		
Mean (BA × TDZ)	0.0	15.2	67.0	70.6	67.9		
	0.5	65.2	66.1	69.7	70.6		
	1.0	71.4	69.7	73.2	74.1		
	2.0	70.5	70.5	74.1	74.1		
Mean (TDZ)		55.6	68.3	71.9	71.7		
Mean (BA)		55.1	67.9	72.1	72.3		

The least significant difference (LSD) for means at significant level 0.05

Factors	NAA	BA	TDZ	NAA × BA	NAA × TDZ	BA × TDZ	NAA × BA × TDZ
LSD 0.05	7.40	3.01	2.99	5.98	5.95	5.95	11.91

NAA – naphthalene acetic acid; BA – benzyl adenine; TDZ – thidiazuron

2005), and that of TDZ may be due to its inhibition of phenolic compounds (Table 1).

Effect of plant growth regulators

The type and concentration of growth regulators are important factors for the in *vitro* regeneration from rose leaf discs (Table 2). No callus was formed on media free of growth regulators. Auxin was found to be important for callus formation regardless of the cytokinins concentration (Fig. 1). In the presence of cytokinins, the rate of callus formation percentage increased with increasing concentration of NAA. Callus production also increased with increasing cytokinins where more leaf discs formed callus with 1 and 2 mg/l of either BA or TDZ. The presence of cytokinin was also essential for callus formation where no callus was observed on media free of both BA and TDZ. However, auxin seems to be more important than cytokinin as some leaf discs produced callus with 2 mg/l NAA alone. The importance of auxin comes from its role

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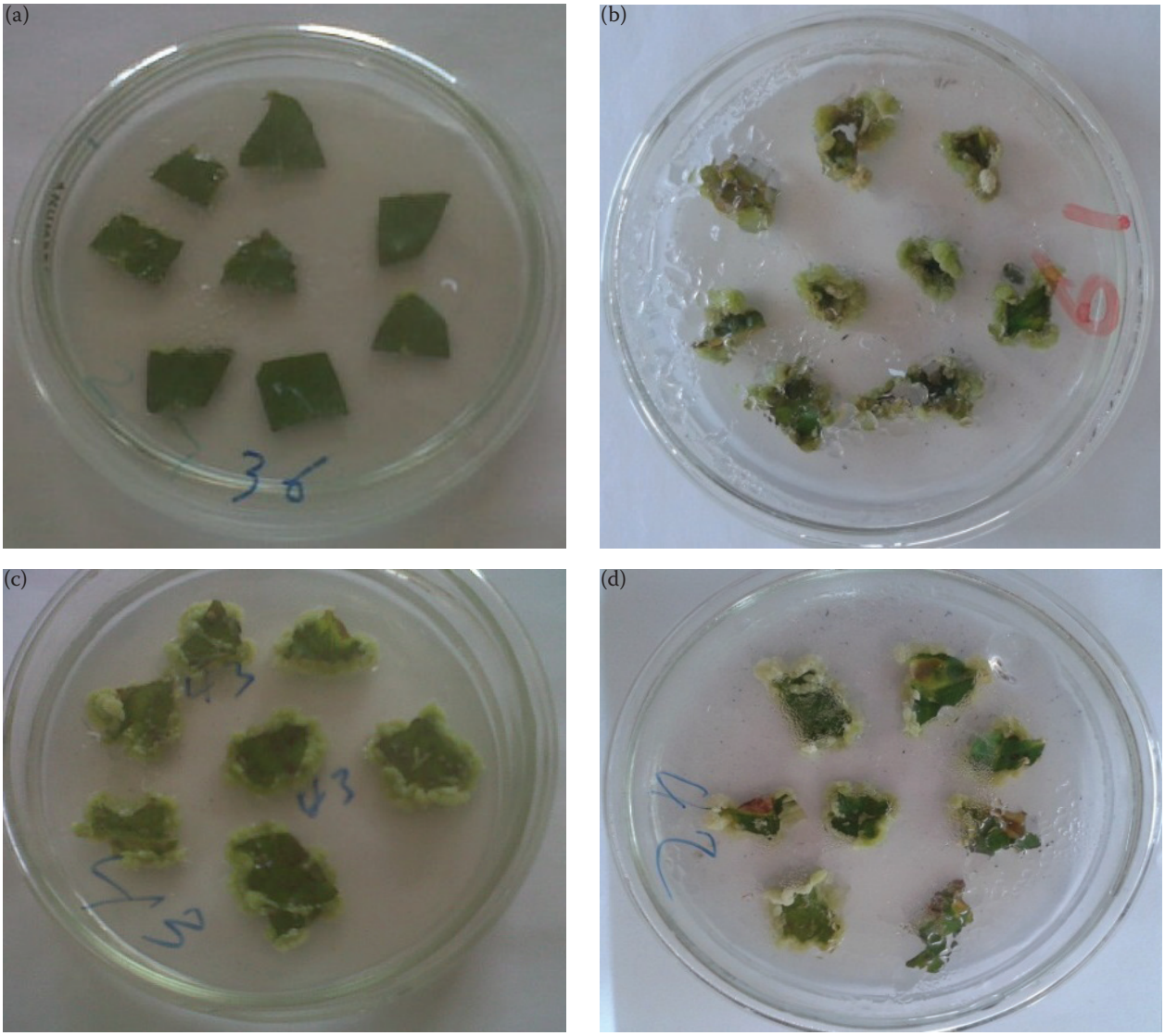

Fig. 1. Callus formation from *in situ* leaf discs of hybrid tea rose ("Eiffel Tower")

(a) no response on media free of growth regulators, (b) callus formation on media containing PGR combinations, (c) callus formed under light condition, (d) callus formed under dark condition

in cell division (Mather, Roberts 1998). The combination between auxin and cytokinin at any concentration gave good response, however, the best results were obtained when NAA was combined with both cytokinins (BA and TDZ). Most leaf discs formed callus on media containing the three growth regulators and the least percentage was recorded on media containing the minimum levels; 0.5 mg/l of NAA in combination with 0.5 mg/l of BA and/or TDZ. Our earlier studies showed the importance of the combination between auxin and cytokinin in plant regeneration from leaf discs and protoplasts of pelargoniums (Hassanein, Dorion 2005, 2006). The presence of auxin with cytokinins was also found to be essential for the regeneration efficiency of other ornamental and medicinal plants (Mendi et al. 2009; Mun, Mun 2016). In the present study, all leaf discs produced good callus but no shoot regeneration was observed (Fig. 1). So, the combination of the three growth regulators was studied under different culture conditions.

Effect of culture conditions (dark and light)

Higher callus formation percentage was obtained in dark compared to light conditions (Table 3). Callus that formed under light was compact and green in color compared to less compact and white

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Table 3. Effect of culture conditions (dark or light) on callus formation (%) from leaf discs of *R. hybrid* cv. "Eiffel Tower", cultivated on different combinations of plant growth regulators (PGR)

PGR combinations (mg/l)			Culture conditions		Mean (PGR combinations)
NAA	BA	TDZ	Light	Dark	
0.5	0.5	0.5	57.1	81.0	69.1
0.5	0.5	1.0	76.2	76.2	76.2
0.5	0.5	2.0	85.7	85.7	85.7
0.5	1.0	0.5	81.0	90.5	85.8
0.5	1.0	1.0	85.7	90.5	88.1
0.5	1.0	2.0	90.5	90.5	90.5
0.5	2.0	0.5	95.2	95.2	95.2
0.5	2.0	1.0	90.5	100.0	95.3
0.5	2.0	2.0	100.0	95.2	97.6
1.0	0.5	0.5	76.2	85.7	81.0
1.0	0.5	1.0	85.7	76.2	81.0
1.0	0.5	2.0	85.7	90.5	88.1
1.0	1.0	0.5	90.5	90.5	90.5
1.0	1.0	1.0	95.2	100.0	97.6
1.0	1.0	2.0	95.2	100.0	97.6
1.0	2.0	0.5	90.5	90.5	90.5
1.0	2.0	1.0	100.0	95.2	97.6
1.0	2.0	2.0	100.0	100.0	100.0
2.0	0.5	0.5	81.0	90.5	85.8
2.0	0.5	1.0	90.5	90.5	90.5
2.0	0.5	2.0	90.5	100.0	95.3
2.0	1.0	0.5	90.5	95.2	92.9
2.0	1.0	1.0	95.2	95.2	95.2
2.0	1.0	2.0	100.0	100.0	100.0
2.0	2.0	0.5	100.0	100.0	100.0
2.0	2.0	1.0	100.0	95.2	97.6
2.0	2.0	2.0	100.0	100.0	100.0
Mean (culture conditions)			89.9	92.9	
The least significant difference (LSD) for means at significant level 0.05					
Factors			Culture conditions	PGR combinations	Conditions × PGR
LSD 0.05			2.62	8.42	18.42

callus under dark conditions (Fig. 1). Less callus formation under light conditions may be related to the degradation of light sensitive PGRs (Mather, Roberts 1998). Similar results on the enhancing effect of dark were previously reported in *Pelargonium* (Hassanein, Dorion 2005) and *R. multiflora* (Canli 2003). All PGRs combinations induced callus formation of leaf discs with the least callus formation on media containing 0.5 or 1 mg/l of both NAA and TDZ in combination with 0.5 mg/l BA. The interaction between PGR and culture conditions was also important where callus formation percentage varied from 57% with the minimum level (0.5 mg/l) of the three PGRs under light to 100% with the combinations containing high levels of cytokinins under either dark or light. The results showed that 0.5 mg/l of NAA in PGR combinations, containing higher concentration of cytokinins, is sufficient for maximum callus formation however no shoot was regenerated. Therefore, PGR combina-

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Table 4. Effect of explants source (*In vitro* or *In situ* mother plants) and growth regulators combination on response and shoot regeneration from leaf discs of *R. hybrid* cv. "Eiffel Tower"

PGR combinations (mg/l)				Response (callus or embryos) (%)			Shoots regeneration (%)	
NAA	BA	TDZ	GA_3	*In situ*	*In vitro*	Mean	*In situ*	*In vitro*
0.5	0.5	0.5	–	80.0	83.3	81.7	–	–
0.5	1.0	1.0	–	93.3	93.3	93.3	–	–
0.5	2.0	2.0	–	100.0	93.3	96.7	–	–
0.5	0.5	–	0.5	96.7	93.3	95.0	–	–
0.5	0.5	–	1.0	100.0	96.7	98.4	–	36.7 ± 0.6
0.5	0.5	–	2.0	96.7	76.7	86.7	–	–
Mean (explant source)				94.5	89.4			

The least significant difference (LSD) for means of response (%) at significant level 0.05

Factors	Explants source	PGR combinations	Source × PGR	Factors	Explants source
LSD 0.05	4.15	5.83	8.25	LSD 0.05	4.15

tions including 0.5 mg/l NAA with varying concentrations of BA and TDZ were studied with several concentrations of GA_3 and two explant sources.

Effect of explant source and gibberellic acid

Generally, explants taken from mother plants grown under *in situ* conditions showed similar or higher callus formation as compared to those taken from *in vitro* mother plants. However, embryos and shoots were developed only on *in vitro* explants (Table 4). Most tested PGR combinations allowed good callus production except for the treatment with 0.5 mg/l of both NAA and BA in combination with 0.5 mg/l TDZ or 2 mg/l GA_3. Leaf discs of *in situ* mother plants showed callus formation with no regeneration regardless of the type or concentration of growth regulators however, somatic embryos and shoots were regenerated from *in vitro* leaf discs (Fig. 2). *In vitro* explants showed somatic embryogenesis with no shoots on media containing TDZ however, 36.7% of explants regenerated shoots directly on medium containing 0.5 mg/l of NAA and BA in combination with 1 mg/l GA_3 (Table 4 and Fig. 2). All embryogenic callus matured and began to regenerate shoots after transferring to fresh medium containing the same PGR combination. These findings agree with those reported on other cultivars of *R. hybrida* (Kim et al. 2004). Results showed the vital role of using *in vitro* mother plants for plant regeneration. This might be explained by the juvenility of *in vitro* plantlets, the hormonal balance in such plantlets as they are sterile and experience less stress due to treatment needed for surface sterilization. Such explants allowed shoot regeneration from some rose cultivars after incubation or several subcultures (Ibrahim, Debergh 2001; Kim et al. 2004). The age and the source of explants were also important variables in the micropropagation of rose and other ornamental and aromatic plants (Mahmoud et al. 2011; Ozel et al. 2015). In our case, shoots regenerated directly after four weeks of culture, which is more rapid than the reported protocols. The somatic embryos can also develop shoots after two weeks of transfer to medium containing GA_3. The favorable role of GA_3 on direct regeneration from leaf explants was reported on *R. canina* (Moallem et al. 2012).

Shoots elongation and plantlets rooting

The transfer of individual shoots to elongation medium containing both indole acetic acid (IAA) and benzyl adenine (BA) encouraged more shoot elongation and leaf development than the medium free of growth regulators or without BA (Table 5). Leaves in these treatments turned to yellow then died within 15 days. These results indicate the importance of growth regulators, especially cytokinin, for good shoot elongationin rose. However, kinetin was better than BA for the elongation of other cultivars of *R. hybrida* (Ibrahim, Debergh 2001), and zeatin was efficient for *in vitro* multiplication of other horticultural crops (Paprštein, Sedlák 2015). The

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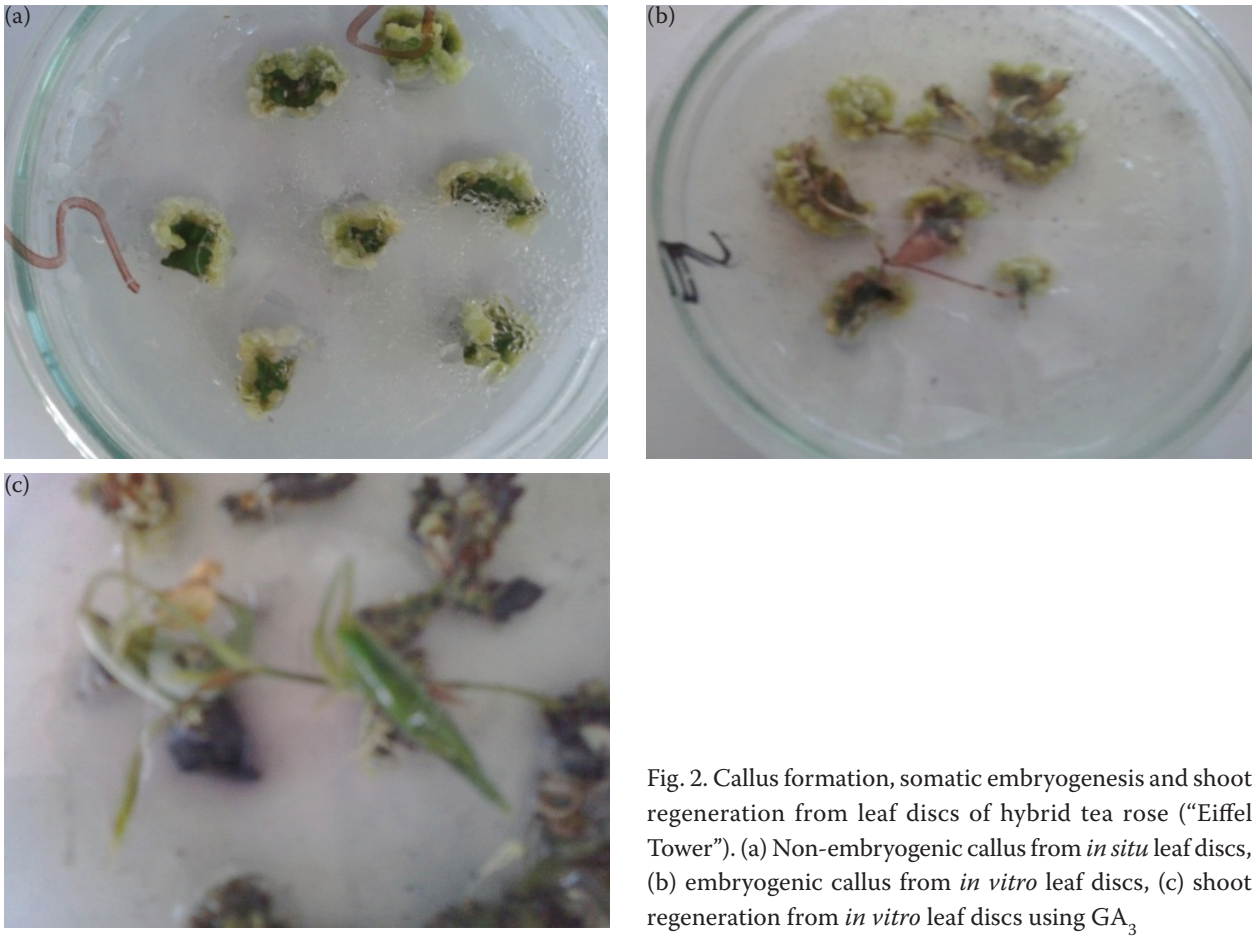

Fig. 2. Callus formation, somatic embryogenesis and shoot regeneration from leaf discs of hybrid tea rose ("Eiffel Tower"). (a) Non-embryogenic callus from *in situ* leaf discs, (b) embryogenic callus from *in vitro* leaf discs, (c) shoot regeneration from *in vitro* leaf discs using GA_3

longest shoots with the most leaves per shoot were obtained with 1 mg/l of IAA and BA (Fig. 3). Similar results were obtained on shoot elongation when IAA was replaced by IBA (data non shown).

No roots were developed on media free of growth regulators or containing IAA whatever its concentration. However, all plantlets rooted on media containing 1 or 2 mg/l IBA, three weeks after transfer (Fig. 3). This result is in agreement with Shekhawat et al. (2015) who found that IBA was the best auxin for rooting of *Passiflora* shoots. It should be mentioned that root tips turned to brown when shoots were kept on rooting medium more than two weeks. This problem was resolved by transferring

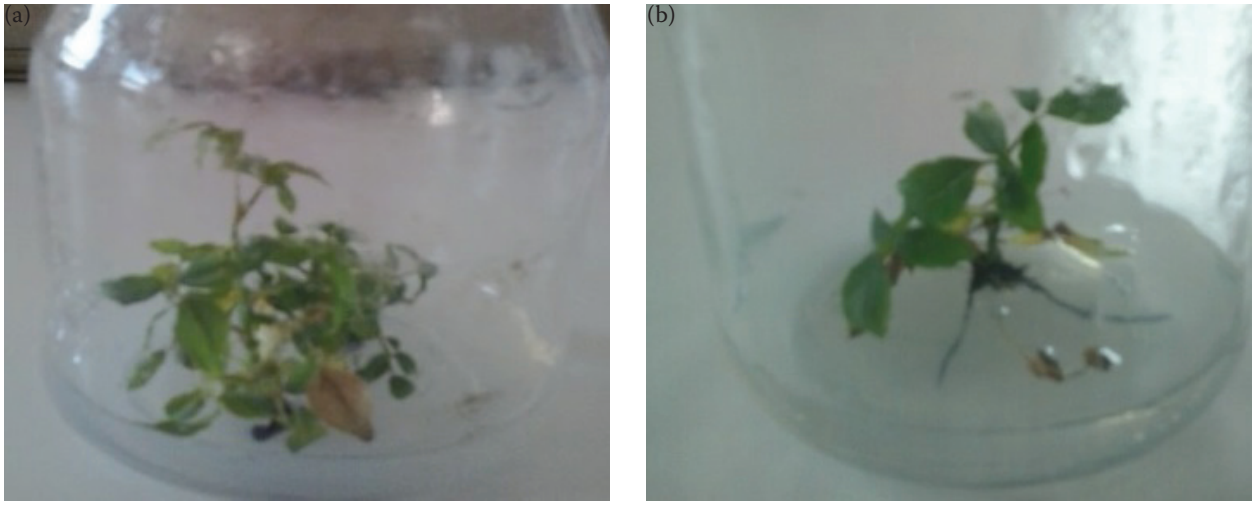

Fig. 3. Shoot elongation (a) and plantlet rooting (b) of hybrid tea rose "Eiffel Tower"

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plantlets to medium free of growth regulators. Similar result was reported on *R. damascene* where auxins were needed for root initiation only but not for subsequent root development (Ginova et al. 2012).

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