Micropropagation of *Rosa rugosa* Through Axillary Shoot Proliferation

Wen Xing, Manzhu Bao, Hongdao Qin, and Guogui Ning[*]

Key laboratory of Horticultural Plant Biology, College of Horticulture and Forestry Sciences, Huazhong Agricultural University, Wuhan 430070, P.R. China

A successful in vitro propagation system has been developed for the valuable ornamental and medicinal plant rugosa rose (*Rosa rugosa* Thunb.) by in vitro culture of nodal segments from mature plants. MS media supplemented with BA 2.2 μM, NAA 0.054 μM, GA_3 2.0 μM and 3% sucrose significantly improved bud break and growth directly from nodal segment explants. High proliferation and average height of shoots were achieved using MS media supplemented with 3% sucrose or glucose adjusted for each cultivar. All the cultivars studied proliferated on MS medium supplemented with BA (2.2 μM), NAA (0.054 μM) and GA_3 (0.4 μM). Rooting of shoots was achieved on half-strength MS medium with IBA (2.5–5.0 μM) combined with glucose or sucrose depending on the cultivar. Plantlets were successfully transferred to pots for acclimatization after they had grown more than 3 roots and average root length exceeded 10 mm.

Key words: *Rosa rugosa*, micropropagation, rooting, acclimatization.

INTRODUCTION

Rugosa rose (*Rosa rugosa* Thunb.) is a deciduous shrub of Rosaceae with ornamental and medicinal value (Jung et al., 2005; Yoshizawa et al., 2000). There is increasing interest in rugosa rose for economic and cultural reasons, including for rose oil and perfume. Especially in East Asian countries this species has been traditionally used in folk medicine to treat several disorders such as diabetes, chronic inflammatory diseases, pain, and cancer (Ng et al., 2004; Park et al., 2005).

For species of the genus *Rosa*, cuttings or grafting onto seedling rootstocks are the predominant techniques but they do not ensure healthy and disease-free plants. Dependence on season and slow multiplication rates are some other major limiting factors (Pati et al., 2006). In vitro propagation has received considerable attention in the last few years. The most important technique of micropropagation, reported by various researchers, is meristem proliferation in which apical buds or nodal segments having an axillary bud are cultured to regenerate multiple shoots without any intervening callus phase (Elliott, 1970; Davies, 1980; Bressan et al., 1982; Barve et al., 1984; Douglas et al., 1989; Rout et al., 1990; Yan et al., 1996; Ara et al., 1997; Carelli and

Echeverrigaray, 2002). In spite of many improvements for some cultivars of roses, micropropagation of rugosa rose is rarely reported and shoot proliferation rates are generally low (Yan et al., 1996).

In this paper we describe an optimal protocol for rapid clonal multiplication of several rugosa rose cultivars originating from Pingyin County (Shandong Province, China) through high frequency axillary shoot proliferation from nodal explants. We examine the influence and interaction of growth regulators (BA, NAA) and carbohydrates (sucrose, glucose) in multiplication of four *Rosa rugosa* cultivars.

MATERIALS AND METHODS

PLANT MATERIAL

One-year-old shoots of 'Tang Red,' 'Puce Dragon,' 'Tang White' and 'Purple Branch' cultivars of *Rosa rugosa* were collected from mature plants growing on the campus of Huazhong Agricultural University, Wuhan, China. The cultivars originated from the breeding center of *Rosa rugosa* in Pingyin County, Shandong Province.

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e-mail: whnobleman2004@yahoo.com.cn

Abbreviations: MS – Murashige and Skoog medium; WPM – woody plant medium; NAA – naphthaleneacetic acid; BA – benzyladenine; IBA – indole-3-butyric acid; GA3 – gibberellic acid; PGR – plant growth regulators.

ESTABLISHMENT AND CONDITIONS
OF IN VITRO CULTURE

One-year-old shoots with leaves removed were cut into 4 cm lengths with one or two nodal segments and washed with running water for 30 min. These were then surface-sterilized by immersion in 70% (v/v) ethanol for 45 sec followed by 0.1% (w/v) mercuric chloride ($HgCl_2$) solution for 10–15 min and then washed three times with sterile distilled water. Afterwards, nodal segments 1.0–1.5 cm long were excised aseptically from the shoots and placed in shoot induction media.

Cultures were incubated at 24±2°C under a 14 h photoperiod at light intensity 40 µmol m^{-2} s^{-1} (cool white fluorescent lamps). All culture media contained 7.5 g l^{-1} agar and 30 g l^{-1} sucrose or glucose. PGR supplements were added to the media before autoclaving for 20 min at 121°C. Stock solutions of GA_3 were filter-sterilized and mixed with autoclaved media cooled to 50–55°C. Four explants per flask were incubated in a 75 mm tumbler holding ~30 ml culture media and sealed with a lid. The pH of the medium was adjusted to 5.8–6.0 with 1 mM NAOH prior to autoclaving.

For shoot initiation, sterilized explants were cultured in light on two basic media, MS or WPM, containing different concentrations of BA (2.2, 4.4 µM), NAA (0.054, 0.27, 0.54 µM) and GA_3 (2.0 µM). The media were supplemented with sucrose (30 g l^{-1}). The explants were cultured at light density 40 µmol m^{-2} s^{-1} (cool-white fluorescent lamps) for 3–4 weeks.

After this time, shoots longer than 10 mm emerging from the nodes were excised and transferred on MS supplemented with different concentrations of BA (2.2, 4.4 µM), NAA (0.054, 0.27, 0.54 µM), GA_3 (0.4, 2.0 µM) and 30 g l^{-1} sucrose or glucose. Two experiments were conducted to determine the most suitable medium for each genotype.

In experiment 1, axillary shoots of 4 cultivars were placed on two MS-based media, supplemented with BA (2.2 µM), NAA (0.054 µM), GA_3 (0.4 µM) and either 30 g l^{-1} sucrose or 30 g l^{-1} glucose. The aim of this experiment was to find the right carbon source for each genotype. In experiment 2 we tested the effects of PGR on shoot multiplication. Six combinations of growth regulators BA (2.2, 4.4 µM) and NAA (0.054, 0.27, 0.54 µM)) added to MS were studied. Cultures were subcultured at monthly intervals on fresh media of the same composition until the shoots had elongated to 1 cm for further submultiplication or even longer for rooting.

For rooting, shoots ~2 cm long with ~4 leaves were harvested and transferred to half-strength MS (inorganic salts, 0.1 mg l^{-1}, thiamine HCl, 0.5 mg l^{-1} nicotinic acid, 0.5 mg l^{-1} pyridoxine HCl, 2 mg l^{-1} glycine, 100 mg l^{-1} myo-inositol) supplemented with IBA (0.5, 2.5, 5.0 µM).

TRANSFER OF PLANTLETS TO SOIL

Rooted plantlets (with more than 3 roots and average root length exceeding 10 mm) were removed from the culture flasks, carefully pulled out of the medium and then transplanted to plastic pots containing a mixture of turf soil + garden soil + sand (2:2:1; v/v/v) kept in a growth chamber and maintained at 19°C and 70% humidity under a 14 h photoperiod. After 2 weeks they were transferred from the pots to the greenhouse for further growth for 2 weeks and irrigated with tap water every 4 days.

STATISTICAL ANALYSIS

Four explants were inoculated in three replicates of each treatment. The experiments were done three times for the multiplication stage and twice for the rooting stage. Shoot proliferation coefficients were calculated by dividing the number of shoots grown at the end of the fourth week by the initial number of buds. Average height of shoot clumps was calculated from measurements of shoots at the end of the fourth week. Roots were counted and measured after 4 weeks of culture on rooting media. Data were evaluated by ANOVA. Means were compared with Duncan's test to determine the significance of differences. Two-way ANOVA was performed to establish the effect of genotype and carbon source. Three-way ANOVA was performed to establish the effect of genotype and growth regulator concentration.

RESULTS

SHOOT INITIATION

After approximately one week, most explants turned green and the node began to expand. Visible elongation of the node on shoot induction medium was observed within 10 days. New budding gradually occurred and reached climax at 3 to 4 weeks (Fig. 1 a–d). Basic culture medium had a significant impact on shoot initiation. For all tested cultivars the proliferation frequency was higher and shoots grew more vigorously on MS medium than on WPM, on which stem browning occurred and growth was slower.

Hormones (PGR) played a key role in shoot initiation. For the 'Tang Red' and 'Puce Dragon' cultivars ~40% of the nodal explants showed bud break on the control medium without growth regulators. The best result was with MS medium containing sucrose (30 g l^{-1}), BA (2.2 µM), NAA (0.054 µM) and GA_3 (2.0 µM), on which 84% of the nodal explants produced at least one shoot. For 'Purple Branch' and 'Tang White', 100% of the nodal explants showed bud break and shoot development on all MS media
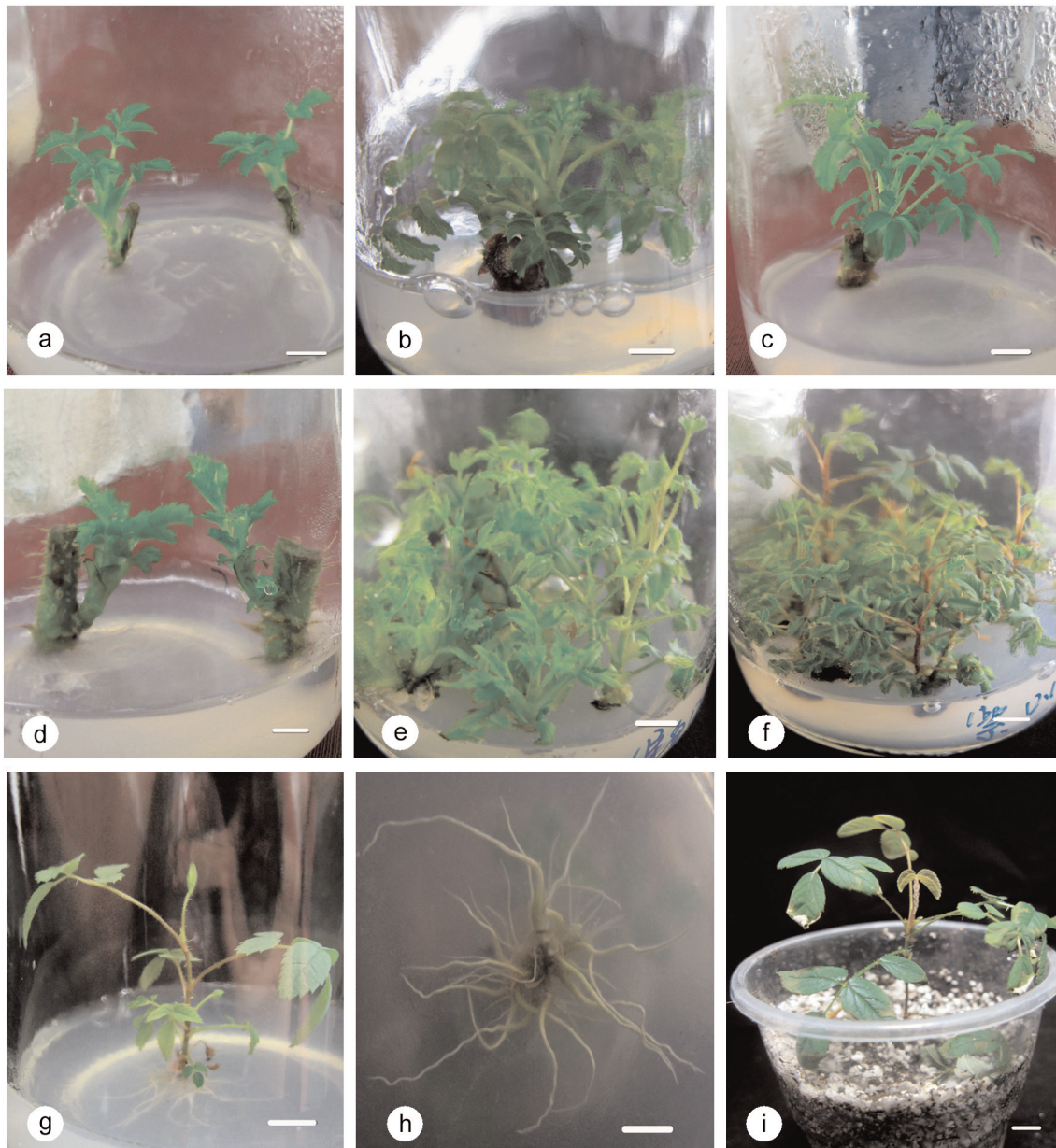
Fig. 1. In vitro culture of four cultivars of *Rosa rugosa* Thunb. Shoot initiation from nodal explants of 'Tang White' **(a)**, 'Purple Branch' **(b)**, 'Puce Dragon' **(c)**, 'Tang Red' **(d)**, Shoot multiplication and elongation of 'Tang White' **(e)**, 'Purple Branch' **(f)**, Rooting, with long roots **(g)**, with large number of small roots; view of bottom of culture tumbler **(h)**, Hardened micropropagated plantlets **(i)**. Bar=0,5 cm.

with or without growth regulators, but the developed shoot became vitrified on the control medium.

EFFECTS OF GENOTYPE AND CARBON SOURCE ON SHOOT MULTIPLICATION

Experiment 1. After 4 weeks, shoots sprouting from the axillary buds were excised from the nodal segments and reintroduced into fresh medium for mul-tiplication. For all genotypes, buds formed from the base and axillary buds of the shoots within 2 weeks (Fig. 1 e, f).

To match the genotype to the proper carbon source for optimal shoot multiplication, we tested all genotypes in MS medium with different carbon sources (30 g l^{-1} sucrose or 30 g l^{-1} glucose) and BA 2.2 μM, NAA 0.054 μM and GA$_3$ 0.4μM. Analysis of variance for proliferation coefficients and average

TABLE 1. Effect of genotype and carbon source on shoot proliferation. Shoots induced from axillary buds of four cultivars were incubated on MS medium supplemented with one of two carbohydrates (sucrose or glucose) combined with BA 2.2 μM, NAA 0.054 μM and GA_3 0.4 μM

Source of variation	df	Mean squares	F value	Pr > F
Genotype	3	0.85	2.83	0.0715
Carbon source	1	4.08	13.56**	0.0020
Genotype* Carbon source	3	2.39	7.96**	0.0018

** Statistically significant at p = 0.05

TABLE 2. Effect of genotype and carbon source on average height of shoot clumps. For explanation see Table 1

Source of variation	df	Mean squares	F value	Pr > F
Genotype	3	1.13	9.00**	0.0010
Carbon source	1	2.04	16.33**	0.0009
Genotype* Carbon source	3	2.54	6.78**	0.0037

** Statistically significant at p = 0.05

height of shoot clumps revealed significant differences between carbon source (p<0.01). The differences between genotypes were significant for average height of shoot clumps but not for proliferation (Tabs. 1, 2). The three cultivars 'Puce Dragon', 'Purple Branch' and 'Tang White' grew better when glucose was used as the carbon source; 'Tang Red' achieved the best growth when sucrose was added to the medium (Tabs. 3, 4).

EFFECTS OF GENOTYPE AND GROWTH REGULATORS ON SHOOTS MULTIPLICATION

Experiment II. Carbon source was an important factor in multiplication. Growth regulators also played a significant role in culture for all the cultivars studied. Axillary shoots developed from nodal culture were harvested and subcultured onto the same shoot initiation medium (MS + BA 2.2 μM + NAA 0.054 μM + GA_3 2.0 μM) to enhance shoot multiplication. The shoots did not grow well and subsequently became necrotic within 2 weeks (data not shown).

ANOVA for proliferation coefficients of shoots (p<0.01) and average height of shoot clumps (p=0.0006) revealed significant differences between genotypes. There were significant differences between genotypes at each BA/NAA concentration. The differences in the interaction between BA and NAA concentration were not significant (p>0.5). ANOVA showed that BA and NAA concentration had significant effects on shoot proliferation and average height of shoot clumps respectively (Tabs. 5, 6).

The effects of BA and NAA concentration on shoot proliferation and average height of shoot clumps are described in Table 7. For all cultivars, shoot proliferation was highest on medium containing 4.4 μM BA, 0.054 μM NAA and 0.4 μM GA_3 but average height of shoot clumps was lower. Shoot proliferation was lower but average height of shoot clumps was higher on medium containing BA 2.2 μM, GA_3 0.4 μM, and NAA 0.27μM or 0.54 μM. For 'Tang Red,' 'Purple Branch' and 'Puce Dragon,' increasing the NAA concentration from 0.27 to 0.54 μM led to the for-

mation of callus. These calli prevented the growth of shoots, leading to necrosis. Considering all the media comprehensively, the best medium for shoot multiplication of each cultivar was MS medium supplemented with BA 2.2 μM, NAA 0.054 μM and GA_3 0.4 μM, on which shoot proliferation (≥3.0) and average height of shoot clumps (>1.3) was greater (Tab. 7).

ROOTING

Individual shoots longer than 10 mm were excised and transferred to a series of rooting media. This experiment examined the influence of different IBA concentrations. When the shoots were incubated on 1/2MS incorporating 0.5–5.0 μM IBA, roots emerged after ~7 days (Fig. 1 g, h). For 'Tang White' there was no difference between IBA concentrations in rooting percentage, number of roots per shoot or root length (p>0.05) (Tab. 8). With the increase of IBA concentration from 0.5 to 5.0 μM, the number of roots per shoot increased, but some plantlets died on 1/2MS supplemented with 2.5 μM IBA. The results were similar for the other cultivars: higher IBA concentrations (2.5–5.0 μM) promoted root formation. At 5.0 μM IBA, the calli formation observed with root initiation inhibited plantlet growth and root elongation (data not shown).

ACCLIMATIZATION AND ESTABLISHMENT OF PLANTS IN SOIL

Rooted plantlets with more than 4 roots longer than 10 mm were transplanted to soil. Using a soil mixture consisting of turf soil + garden soil + sand (2:2:1; v/v/v), more than 80% of the plants were successfully established in the greenhouse after 2–3 weeks (Fig. 1 i).

DISCUSSION

A method of accelerating proliferation is needed for *Rosa rugosa* Thunb., a plant of high economic and medicinal value. The in vitro propagation system reported here should prove an effective and useful procedure for breeding *Rosa rugosa*.

TABLE 3. Shoot proliferation in four *Rosa rugosa* cultivars initiated with different carbon sources. Shoots of four cultivars were cultured on MS medium with sucrose or glucose and with BA 2.2 µM, NAA 0.054 µM and GA$_3$ 0.4 µM

Carbon source	Genotype			
	'Tang Red'	'Puce Dragon'	'Purple Branch'	'Tang Whilte'
Sucrose	3.97±0.58a	2.76±0.68a	3.33±0.35a	2.60±0.53a
Glucose	3.00±0.30b	3.63±0.60b	4.87±0.51b	4.47±0.70b

Data were recorded at the end of four weeks.Values shown are means ±SE.
Values with different letters within columns differ significantly at p=0.05.

TABLE 4. Average height of shoot clumps of four *Rosa rugosa* cultivars initiated with different carbon sources. For explanation see Table 3

Carbon source	Genotype			
	'Tang Red'	'Puce Dragon'	'Purple Branch'	'Tang Whilte'
Sucrose	1.50±0.50a	1.83±0.29a	1.67±0.29a	2.00±0.50a
Glucose	2.16±0.29b	1.63±0.29b	2.67±0.29b	3.16±0.29b

Data were recorded at the end of the fourth week. Values shown are means ±SE.
Values with different letters within columns differ significantly at p=0.05.

TABLE 5. Effect of genotype and BA/NAA concentration in culture medium on average height of shoot clumps. Shoots of four cultivars were incubated on MS medium supplemented with different concentrations of BA (2.2 µM, 4.4 µM) and NAA (0.054 µM, 0.27 µM, 054 µM)

Source of variation	df	Mean squares	F value	Pr > F
Genotype	3	9.18	87.39**	<.0001
BA	2	10.03	95.48**	<.0001
NAA	1	0.49	4.68**	0.0141
BA* NAA	2	0.06	0.58	0.5631
Genotype*BA*NAA	6	0.75	7.77**	<.0001

[**] Statistically significant at p = 0.05

TABLE 6. Effect of genotype and BA/NAA concentration in culture medium on shoot proliferation. For explanation see Table 5

Source of variation	df	Mean squares	F value	Pr > F
Genotype	3	3.03	6.84**	0.0006
BA	2	0.24	0.55	0.4606
NAA	1	24.78	56.01**	<.0001
BA* NAA	2	0.09	0.21	0.8086
Genotype*BA*NAA	6	0.85	1.93**	<.0001

[**] Statistically significant at p = 0.05

TABLE 7. Proliferation and average height of shoot clumps of four *Rosa rugosa* cultivars under different BA and NAA concentrations. Shoots of four cultivars were cultured on multiplication medium containing 3% glucose, GA$_3$ 0.4 µM and different concentrations of BA and NAA

Growth regulators µM			Cultivar							
			'Tang White'		'Purple Branch'		'Puce Dragon'		'Tang Red'	
BA	NAA	GA$_3$	Proliferation coefficients	Average height of shoot clumps	Proliferation coefficients	Average height of shoot clumps	Proliferation coefficients	Average height of shoot clumps	Proliferation coefficients	Average height of shoot clumps
4.4	0.054	0.4	4.23±0.68ab	2.67±0.58b	4.00±0.89a	1.16±0.29c	3.87±0.51a	1.33±0.58b	3.20±0.17a	1.00±0.00b
4.4	0.27	0.4	2.96±0.46bc	2.00±0.50c	1.67±1.15b	1.33±0.29c	2.00±0.30b	1.50±0.00a	1.77±0.68b	1.67±0.29a
4.4	0.54	0.4	3.13±1.25bc	2.00±0.00c	2.33±0.35ab	1.83±0.29b	1.77±0.50b	2.00±0.00a	1.33±0.58b	1.50±0.00ab
2.2	0.054	0.4	4.47±0.70a	3.17±0.29b	3.97±0.58a	2.67±0.29a	3.67±0.65a	2.17±0.29a	3.00±0.30a	1.33±0.29ab
2.2	0.27	0.4	1.57±0.23d	4.16±0.29a	2.97±0.53ab	2.00±0.00b	1.80±0.70b	1.67±0.29a	1.50±0.23b	1.50±0.50ab
2.2	0.54	0.4	2.13±0.51cd	4.16±0.29a	1.90±0.17b	2.00±0.00b	1.67±0.53b	2.00±0.50a	1.90±0.17b	1.67±0.29a

Data were recorded at the end of the fourth week. Values shown are means ±SE.
Values with different letters within columns differ significantly at p = 0.05.

TABLE 8. Effect of IBA concentration in culture medium on rooting of 'Tang White' cultivar after 3 weeks of culture. Shoots longer than 10 mm were cultured on 1/2MS medium supplemented with different concentrations of IBA

IBA (μM)	Rooting (%)	No. of roots/ shoot	Root length (mm)
0.5	60.00±41.83a	2.00±1.50 a	2.88±0.85 a
2.5	80.00±44.72a	4.10±3.05 a	2.50±1.29a
5.0	80.00±27.39a	4.10±0.89 a	2.80±0.84a

Data were recorded at the end of the third week. Values shown are means ±SE. Values with different letters within columns differ significantly at p = 0.05.

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 plants raised from tissue culture (Pati et al., 2006). Our work placed stress on those micropropagation stages and also paid more attention to the culture factors promoting successful shoot multiplication, such as medium, carbon source and growth regulators.

For initiation of aseptic cultures in the genus *Rosa*, the explants commonly used are nodal stem segments, the axillary buds of which were found to be good for proliferating multiple shoots (Pati et al., 2006). We had the same results in our studies: each nodal segment could form at least one or two shoots. The basic culture medium also had a significant effect on culture initiation. WPM is a widely used culture medium for woody plants (Van der Salm et al., 1993) but stem of rugosa rose cultured on WPM medium underwent severe browning during the culture process, which inhibited budding break and growth. On MS medium only slight browning occurred and the initiated shoots were much stronger. These results have been documented in micropropagation of other rose species as well (Yan et al., 1996). In previous studies on the effect of cytokinins and auxins on initiation of aseptic cultures from rose nodal stem segments (Davies, 1980; Vijaya et al., 1991; Carelli and Echeverrigaray, 2002), BA (4.4–13.2 μM/l) as a major PGR or in combination with low NAA concentration (0.0216–0.54 μM/l)) was found suitable for shoot initiation. Other researchers have reported a high percentage of bud break on hormone-free medium within 10–12 days, but the growth rate was very low in roses (Rout et al., 2004). In our study the effects of different concentrations of BA (2.2–4.4 μM/l) and NAA (0.054–0.54 μM/l) on bud break and growth were not obvious for all cultivars; explants grew well at different PGR concentrations without visible morphological abnormalities but also without increased proliferation, but on MS medium without growth regulator the growth rate was lower and the induced shoots were seriously vitrified.

For shoot multiplication, sucrose (3%) has commonly been used as carbon source. Murashige and Skoog (1962) stated that 3% sucrose is generally better than 2% or 4% for tissue culture. In our work, glucose was better than sucrose for 'Puce Dragon', 'Purple Branch' and 'Tang White', yielding higher proliferation and taller shoots. On medium supplemented with 3% sucrose almost all the new leaves turned brown, which inhibited shoot growth.

In vitro shoot multiplication relies largely on medium formulations containing BA as the major PGR in combination with a low concentration of NAA (Syamal and Singh, 1996; Singh and Syamal, 1999; Carelli and Echeverrigaray, 2002). In the present study, 2.2 μM/l BAA in combination with low NAA (0.054 μM/l) was the optimum treatment for in vitro multiplication of rugosa rose; this result differs from other findings on optimal BA concentration (4.4–13.2 μM/l) (Carelli and Echeverrigaray, 2002; Singh and Syamal, 1999; Davies, 1980). BA is needed for proliferation of rugosa rose plantlets, but a high concentration is undesirable: at a concentration higher than 2.2 μM it will lead to multiplication of shoots, which is not beneficial to shoot elongation. When the culture medium contains NAA at higher concentration (0.27 μM) the bud can form more callus from the base section, which will greatly affect the young seedling's absorption of water and nutrition, and thus inhibit its growth. The inhibition is especially obvious when NAA concentration is increased to 0.54 μM. A high concentration of GA_3 (2.0 μM) always finally caused the nigrescence and death of young shoots, which appeared waterlogged.

Rooting of multiplied rugosa rose shoots was achieved on medium with IBA alone. The best results were with 1/2MS medium with IBA 2.5 or 5.0 μM. The same results were reported in *R. hybrida* cv. Peace (Kirichenko et al., 1991).

Our study yielded a practical protocol for efficient axillary bud multiplication from rugosa rose explants. Following upon previous work by others (Yan et al., 1996), here we demonstrate high-efficiency micropropagation of rugosa rose for the first time. Micropropagation of rugosa rose provides an opportunity to harvest virus-free material and conserve important germplasm resources in vitro. It furnishes material for genetic transformation and for employing molecular techniques in breeding.

ACKNOWLEDGEMENTS

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