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In vitro flowering red miniature rose

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Abstract

Using aseptic plantlets obtained from stem node explants of hybrid red miniature rose (*Rosa hybrida* cv. Fairy Dance), the effects of shoot physiological status, medium ingredients, and culture thermoperiod on *in vitro* flowering were evaluated. Shoot height, subculture media for shoot multiplication, sucrose concentration, plant growth regulators (PGRs), mineral substances in media, and thermoperiod had a significant effect on the percentage of *in vitro* flowering. Shoots 3 ± 0.2 or 2 ± 0.2 cm in height cultured on Murashige and Skoog (MS) medium containing 2.0 mg dm^{-3} 6-benzyladenine (BA), 0.2 mg dm^{-3} α-naphthaleneacetic acid (NAA), and 20 g dm^{-3} sucrose were more suitable for *in vitro* flowering than shoots 4 ± 0.2, or 5 ± 0.2 cm in height. The most suitable sucrose concentration for *in vitro* flowering was 50 g dm^{-3} and the most suitable PGRs were a combination of 3.0 mg dm^{-3} BA and 0.1 mg dm^{-3} NAA. Increasing the potassium nitrate to ammonium nitrate ratio or increasing the phosphate concentration in MS medium had a positive effect on *in vitro* flowering. The percentage of *in vitro* flowering was significantly higher at day/night temperature of 28/20 °C than at other constant temperatures. The percentage of *in vitro* flowering shoots reached 68.33 % despite the occurrence of abnormal flowers and some unique developmental patterns. It makes miniature rose a potentially new *in vitro* experimental platform for research on the molecular mechanisms of flowering ornamental plants.

Additional key words: abnormal flower, auxins, cytokinins, minerals, *Rosa hybrida*, thermoperiod.

Introduction

Rose is one of the world most popular ornamental flowers because of its varied colours, beautiful shapes, attractive smell, and a long-lasting inflorescence reflecting complex breeding systems and pollination relationships (MacPhail and Kevan 2009). Nowadays, roses are grown world-wide as cut flowers, potted plants, and for garden decoration (Vu *et al.* 2006). Flower formation *in vitro* can provide a model system for flower initiation and development and can be further applied for *in vitro* breeding due to the high occurrence of mutations which are useful, especially for those plants with long periods of vegetative growth. Simultaneously, flowering miniaturized plantlets *in vitro* has a good commercial potential for the ornamental trade (Wang *et al.* 2002). The terms test tube or *in vitro* bouquets were coined specifically for *in vitro* flowering ornamental plants (Sudhakaran and Sivasankari 2002, Sudhakaran *et al.* 2006). To date, there are many studies on *in vitro* flowering plants. The effects of shoot physiological status, medium ingredients, and culture conditions on *in vitro* floral morphogenesis are species-specific (Taylor and Van Staden 2006, Mondal *et al.* 2011).

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Abbreviations: BA - 6-benzyladenine; DMRT - Duncan's multiple range test; IBA - indole-3-butyric acid; KIN - kinetin; MS - Murashige and Skoog medium; NAA - α-naphthaleneacetic acid; PGR - plant growth regulator; PPFD - photosynthetic photon flux density; TDZ - thidiazuron; ZT - zeatin.
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The ability of explants to form flowers *in vitro* depends on numerous factors internal and external, chemical and physical, all of which virtually interact in various complex and unpredictable ways (Teixeira da Silva and Nhut 2003, Vu *et al.* 2006, Deb and Sungkumlong 2009, Wang *et al.* 2009, Chiu *et al.* 2011, Wilmowicz *et al.* 2011). Thus, *in vitro* conditions can be manipulated to provide an alternative controlled system necessary to study flower induction, inflorescence, and flower morphogenesis. *In vitro* flowering can also be applied as a tool to accelerate breeding programs or can be adjusted to the commercial production of specific compounds from floral organs (Ziv and Naor 2006). The physiological stage of the mother plant and the organ or tissue source of the explant, media components, level of plant growth regulators (PGRs), and culture conditions significantly affect *in vitro* flowering. In most *in vitro* flowering studies, cytokinins such as 6-benzyladenine (BA), zeatin (ZT), thidiazuron (TDZ), and kinetin (KN), or combinations of PGRs and nutrients have been used to induce *in vitro* flowering (Kintzios and Michaelakis 1999, Luo *et al.* 2000).

Despite many studies on the micropropagation of rose (Carelli *et al.* 2002, Pati *et al.* 2006, Khosh-Khui and Teixeira da Silva 2006), there are a few reports on *in vitro* flowering (Mohapatra *et al.* 2005, Wang *et al.* 2002, Vu *et al.* 2006). In those studies, PGRs and their concentrations played an important role in the induction of *in vitro* flowers of rose although the percentage was dependent on cultivars.

In the present study, an attempt was made to determine the most favorable environmental and nutrition factors for *in vitro* flowering of red miniature rose such as shoot height, culture medium for bud multiplication, sucrose concentration, PGRs, mineral substances in culture media, and thermoperiod.

Materials and methods

Hybrid red miniature rose plants (*Rosa hybrida* cv. Fairy Dance) were maintained in a greenhouse of the South China Botanical Garden, Guangzhou, China (Fig. 1*A*). Three hundred nodal sections (2 - 3 cm in height and containing dormant vegetative axillary buds) were excised as explants to establish the micropropagation system. The nodal sections were surface sterilized three times with a 0.1 % (m/v) $HgCl_2$ solution containing 0.1 % (v/v) *Tween-20*, each time for 3 min. The explants were then rinsed 5 times with sterile distilled water. Finally, dormant buds with a small amount of surrounding tissue were aseptically separated from the nodal sections and placed vertically in a natural orientation on Murashige and Skoog (1962; MS) medium supplemented with 3.0 mg dm^{-3} BA and 0.2 mg dm^{-3} α-naphthaleneacetic acid (NAA) to induce the sprouting of axillary buds.

After 30 d in primary culture, the axillary buds were transferred to multiplication media to mass proliferation of shoots. Following initial trials, the media tested were MS basal medium supplemented with BA (0.2, 0.5, 1.0, 1.5, 2.0, and 3.0 mg dm^{-3}) in combination with 0.2 mg dm^{-3} NAA. Plant growth regulators (PGRs) were purchased from *Sigma* (St. Louis, USA). All media were supplemented with 20 g dm^{-3} sucrose and 6 g dm^{-3} agar. The pH of culture media was adjusted to 5.8 before autoclaving at 121 °C and 1.06 kg cm^{-2} for 20 min. All cultures were incubated in a growth chamber at 26 ± 2 °C under a 12-h photoperiod (cool white fluorescent lamps, photosynthetic photon flux density (PPFD) of 35 ± 5 μmol m^{-2} s^{-1}. Multiplication of shoots by subcultures was performed at 50-d intervals. The multiplication ratio was calculated in the 5th subculture as the number of new buds that formed out of per explant cultured in flasks after 50 d in culture. Each treatment contained three replicates with 10 flasks per replicate and 20 plantlets per flask, and means ± SE of 600 replicates were calculated.

Following initial trials, shoots 2 ± 0.2, 3 ± 0.2, 4 ± 0.2, or 5 ± 0.2 cm in height derived on multiplication medium A in the 5th subculture (MS supplemented with 0.5 mg dm^{-3} BA, 0.2 mg dm^{-3} NAA, and 20 g dm^{-3} sucrose) or shoots 1 ± 0.2, 2 ± 0.2, 3 ± 0.2, or 4 ± 0.2 cm in height derived from multiplication medium B in the 5th subculture (MS supplemented with 2.0 mg dm^{-3} BA, 0.2 mg dm^{-3} NAA, and 20 g dm^{-3} sucrose) were cultured on MS medium containing 3.0 mg dm^{-3} BA, 0.1 mg dm^{-3} NAA, and 50 g dm^{-3} sucrose. The percentage of *in vitro* flowering was calculated as the number of flowering shoots out of the total number of cultured shoots in a flask after 60 d in culture. Each treatment contained three replicates with 10 flasks per replicate and 20 plantlets per flask.

Further, individual shoots (2 - 3 cm in height) were collected from medium B (these shoots were the most suitable for *in vitro* flowering according to preliminary experiments) were transferred to MS medium containing 3.0 mg dm^{-3} BA, 0.1 mg dm^{-3} NAA, and various concentrations of sucrose (0, 10, 20, 30, 40, 50, or 60 g dm^{-3}).

Similarly, individual shoots (2 - 3 cm in height) collected from medium B were transferred to MS media containing 3.0 mg dm^{-3} BA, 0.1 mg dm^{-3} NAA, and different concentrations of N, P, and K which were obtained by reducing or increasing the amount of potassium nitrate (KNO_3), ammonium nitrate (NH_4NO_3), and potassium phosphate (KH_2PO_4) (Table 4).

To test the effect of PGRs, individual shoots (2 - 3 cm in height) collected from medium B were transferred to media containing 0.1, 0.2, 0.5, 1.0, and 2.0 mg dm^{-3} TDZ; 0.5, 1.0, 2.0, 3.0, and 4.0 mg dm^{-3} BA; 0.1, 0.2, 0.5, 1.0, and 2.0 mg dm^{-3} ZT; 0.5, 1.0, 3.0, 5.0, and 7.0 mg dm^{-3} kinetin (KIN) alone or together with NAA or indole-3-butyric acid (IBA) at concentrations of 0.1 or 0.2 mg dm^{-3}.

To study the effect of temperature, individual shoots (2 - 3 cm in height) collected from medium B were transferred to MS medium containing 3.0 mg dm^{-3} BA and 0.1 mg dm^{-3} NAA at constant temperature of 24, 26, 28, 30, and 32 °C or at day/night temperatures of 28/20 °C.

All experiments were established in a completely randomized design. All experiments were repeated three times. In addition, each treatment contained three replicates with 10 flasks per replicate and 20 plantlets per flask, and means ± SE of 600 replicates were calculated. Data were analyzed with *SPSS 11.0* for *Windows* using one-way *ANOVA* followed by Duncan's multiple range test (DMRT) to test for significant differences between means at $P \leq 0.05$.

Results

Most explants (88 %) were free from contamination after sterilization. Axillary buds appeared within about 15 d of culture. To ensure the unity of the material, shoots 2 to 3 cm in height were cut into single nodes and subcultured onto different multiplication media which had significant effects on shoot height and multiplication rate. Within the tested BA concentrations, as the concentration increased, multiplication rate increased too whereas shoot height decreased (Table 1, Fig. 1*C*). The shoots grew normally and were thus suitable for the further experiments. Only when 3.0 mg dm^{-3} BA was used, the shoots were short and thin with many clustered buds, some of them abnormal.

Table 1. Effect of BA in MS basal medium containing 20 g dm^{-3} sucrose and 0.2 mg dm^{-3} NAA on *in vitro* multiplication of miniature hybrid rose (*Rosa hybrida* cv. Fairy Dance) determined after 50 d of culture. Means ± SE of 600 replicates; different letters within a column indicate significant differences at $P \leq 0.05$ according to DMRT.

BA [mg dm^{-3}]	Shoot height [cm]	Multiplication ratio
0	4.53 ± 1.20 e	1.25 ± 0.21 g
0.2	5.35 ± 0.17 f	2.27 ± 0.21 f
0.5	4.62 ± 0.20 e	2.93 ± 0.15 e
1.0	4.37 ± 0.18 d	3.27 ± 0.15 d
1.5	3.54 ± 0.10 c	3.73 ± 0.17 c
2.0	2.85 ± 0.24 b	4.30 ± 0.20 b
3.0	2.32 ± 0.08 a	5.33 ± 0.13 a

Shoot height and medium type affected the percentage of *in vitro* flowering (Table 2). The highest percentage of *in vitro* flowering was 52.33 % from 2-cm high shoots growing on medium B. This percentage was significantly higher than that from shoots 1 or 4 cm high or from shoots of all heights on medium A. The *in vitro* flowers had about half the diameter of *in vivo* flowers (Fig. 1*F*).

Sucrose concentration had a significant influence on *in vitro* flowering (Table 3). MS medium containing 50 g dm^{-3} sucrose resulted in the higher percentage of *in vitro* flowering than on media with other sucrose concentrations. No shoots flowered *in vitro* when the sucrose concentration was lower than 20 g dm^{-3}.

Also mineral content had a significant influence on *in vitro* flowering red miniature rose (Table 4). The percentage of *in vitro* flowering was significantly higher

Table 2. Effect of shoot height and different multiplication medium during cultivation of explants on *in vitro* flowering miniature hybrid rose after 60 d of culture on MS medium containing 3.0 mg dm^{-3} BA, 0.1 mg dm^{-3} NAA, and 50 g dm^{-3} sucrose. Medium A: MS + 0.5 mg dm^{-3} BA, 0.2 mg dm^{-3} NAA, and 20 g dm^{-3} sucrose; medium B: MS + 2.0 mg dm^{-3} BA, 0.2 mg dm^{-3} NAA, and 20 g dm^{-3} sucrose. Means ± SE of 600 replicates; different letters within a column indicate significant differences at $P \leq 0.05$ according to DMRT.

Media	Shoot height [cm]	Flowering [%]
A	5 ± 0.2	27.67 ± 1.45 c
	4 ± 0.2	36.67 ± 1.76 b
	3 ± 0.2	39.33 ± 2.33 b
	2 ± 0.2	43.00 ± 2.64 b
B	4 ± 0.2	40.67 ± 1.45 b
	3 ± 0.2	49.00 ± 2.08 a
	2 ± 0.2	52.33 ± 1.45 a
	1 ± 0.2	30.67 ± 1.76 c

Table 3. Effect of sucrose concentration on *in vitro* flowering miniature hybrid rose after 60 d of culture on MS basal medium containing 3.0 mg dm^{-3} BA and 0.1 mg dm^{-3} NAA. Means ± SE of 600 replicates; different letters within a column indicate significant differences at $P \leq 0.05$ according to DMRT.

Sucrose concentration [g dm^{-3}]	Flowering [%]
0	0
10	0
20	0
30	35.00 ± 1.73 d
40	47.00 ± 1.15 b
50	52.33 ± 1.45 a
60	42.67 ± 2.02 c

on MS medium containing 1.9 g dm^{-3} KNO_3 and 1.65 g dm^{-3} NH_4NO_3 than on MS medium supplemented with 3.8 g dm^{-3} KNO_3 and 3.3 g dm^{-3} NH_4NO_3, or MS medium supplemented with 0.95 g dm^{-3} KNO_3 and 0.82 g dm^{-3} NH_4NO_3 but was not significantly different from MS medium supplemented with 2.85 g dm^{-3} KNO_3 and 2.47 g dm^{-3} NH_4NO_3. When the ratio of KNO_3 to NH_4NO_3 was 1:3.45 or 1:4.84 (m/m), the percentage of *in vitro* flowering was significantly lower than in the

control (in which the ratio was 1:6.59). When the ratio of KNO_3 to NH_4NO_3 was 1:13.11 or 1:16.83, the percentage of *in vitro* flowering was significantly higher than in the control. When the medium did not contain KH_2PO_4, no *in vitro* flowering occurred but it increased with increasing KH_2PO_4 concentration (Table 4).

PGRs significantly influenced the *in vitro* flowering red miniature rose (Table 5). There was no *in vitro* flowering on MS media without cytokinins and only with auxin (NAA or IBA). Among all the tested cytokinins (TDZ, BA, ZT, and KIN), MS medium with 3.0 mg dm^{-3} BA was most effective for *in vitro* flowering (Fig. 1*D*,*E*,*F*); the percentage of *in vitro* flowering shoots on this medium was significantly higher than on every other medium. Noticeably, many abnormal flowers were observed, especially on MS medium containing 2.0 mg dm^{-3} TDZ (about 10 %), including the development of stems and leaves into flowers (Fig. 1*G*), an alabastrum forming on a flower (Fig. 1*H*), red flowers reverting to pink flowers (Fig. 1*I*), an alabastrum forming on white flowers (Fig. 1*J*), flowers without a stamen (Fig. 1*K*), petals changing into leaves (Fig. 1*L*), flowers with fewer petals and without stamens and pistils (Fig. 1*M*), and flowers forming on other

Fig. 1. *In vitro* flowering hybrid red miniature rose (*Rosa hybrida* cv. Fairy Dance). *A* - Flowering red miniature rose growing in pots. *B* - Emerging axillary shoots from explants on MS medium containing 2.0 mg dm^{-3} BA, 0.2 mg dm^{-3} NAA, and 20 g dm^{-3} sucrose. *C* - Proliferation of cluster buds on MS medium containing 2.0 mg dm^{-3} BA, 0.2 mg dm^{-3} NAA, and 20 g dm^{-3} sucrose. *D*,*E* - Flowering *in vitro* on MS medium containing 3.0 mg dm^{-3} BA and 0.1 mg dm^{-3} NAA. *F* - Normal flower *in vitro*. *G* - Stem and leaves developing into flower. *H* - Alabastrum forming onto flower. *I* - Red flower forming inside pink flower. *J* - Alabastrum forming in white flower. *K* - Flower without stamen. *L* - Petals change into leaves. *M* - Flower with fewer petals and without stamen and pistil. *N* - Flower forming inside flower *in vitro*. *Bar* is 20 cm for *A*, 5 cm for *B* to *D*, 20 cm for *E*, and 1 cm for *F* to *N*.

Table 4. Effect of minerals [mg dm^{-3}] in media on *in vitro* flowering [%] miniature hybrid rose after 60 d in culture on modified MS basal medium containing 3.0 mg dm^{-3} BA, 0.1 mg dm^{-3} NAA, and 50 g dm^{-3} sucrose. Means ± SE of 600 replicates; different letters within a column indicate significant differences at $P \leq 0.05$ according to DMRT.

NH_4NO_3	KNO_3	KH_2PO_4	$[NH_4^+]$	$[NO_3^-]$	$[NH_4^+]$:$[NO_3^-]$	Total N	$[PO_4^{3-}]$	$[K^+]$	Flowering
1650	1900	170	371	2445	1: 6.59	841	119	782	52.33 ± 1.45cde
825	950	170	186	1223	1: 6.59	421	119	415	42.33 ± 2.06 g
2475	2850	170	557	3664	1: 6.59	1263	119	1149	51.00 ± 2.65 defg
3300	3800	170	743	2468	1: 6.59	1682	119	1514	44.00 ± 3.06 f
0	6068	170	0	3721	-	841	119	2428	19.67 ± 2.60 i
2402	0	170	540	1863	1: 3.45	841	119	49	15.33 ± 2.60 i
2000	1025	170	450	2177	1: 4.84	841	119	444	33.67 ± 1.86 h
1252	2905	170	282	2751	1: 9.76	841	119	1169	55.00 ± 1.73 cd
1000	3542	170	225	2946	1:13.11	841	119	1418	64.67 ± 2.60 ab
825	2943	170	186	3131	1:16.83	841	119	1184	58.67 ± 1.76 bc
1650	1900	0	371	2443	1: 6.59	841	0	733	0 j
1650	1900	85	371	443	1: 6.59	841	60	758	45.67 ± 3.48 efg
1650	1900	340	371	443	1: 6.59	841	225	815	68.33 ± 2.03 a
1650	1900	680	371	443	1: 6.59	841	450	728	58.33 ± 2.03 ab

flowers *in vitro* (Fig. 1*N*). In addition, an appropriate cytokinin concentration (1.0 mg dm^{-3} TDZ or 3.0 mg dm^{-3} BA) combined with 0.1 or 0.2 mg dm^{-3} NAA or 0.1 mg IBA stimulated *in vitro* flowering, although the percentage of *in vitro* flowering was not significantly different when the same media were supplemented with 0.2 mg dm^{-3} IBA which resulted in the rooting of shoots.

Different temperatures had also a significant effect on *in vitro* flowering and 28 °C was the best constant temperature (Table 6). However, different day/night temperatures (28/20 °C) induced the highest percentage of *in vitro* flowering (65.67 %).

Discussion

Different plant species require different induction media to induce *in vitro* flowers (Ziv and Naor 2006). Due to the genotypic diversity of different cultivars, the percentage of *in vitro* flowering differs from one cultivar to another (Douglas *et al.* 1989, Wang *et al.* 2002, Ishimori *et al.* 2009). Wang *et al.* (2009) reported that the total time from an original culture and subculture time before flower induction were two very important factors for *in vitro* flower induction. In the present study, different percentages of *in vitro* flowering were obtained on shoots of different heights from the same multiplication media. This may be because those shoots had a different physiological status or stage whereas different percentages of *in vitro* flowering obtained on shoots of the same height from different subculture media may be due to different levels of endogenous phyto-hormones or other unknown substances in those shoots. The rationale is that some types of compounds, for example proline, can induce flowering together with hormones (Saxena *et al.* 2008).

In vitro flowering is influenced by the two major medium components, sugars and minerals (reviewed by Scorza 1982, Ziv and Naor 2006). Sugars are considered to be necessary carbon sources in culture media for the viable induction and development of flowers, for instance in *Fagopyrum esculentum* (Kachonpadungkitti *et al.* 2001) and *Momordica charantia* L. (Wang *et al.* 2001). Vu *et al.* (2006) suggested that sucrose was the only factor needed for floral bud induction or initial development whereas other factor(s) are important in later stages of *in vitro* floral development. In their study, the most suitable sucrose concentration was 30 g dm^{-3}. In the present study, the most suitable sucrose concentration was 50 mg dm^{-3}. In MS medium, high concentrations of nitrogen usually inhibited flowering and promoted vegetative growth whereas the use of half-strength MS mineral medium or a reduced nitrogen content enhanced *in vitro* flowering in *Cymbidium* (Kostenyuk *et al.* 1999), *Doritis* (Duan and Yazawa 1994), *Phalaenopsis* (Duan and Yazawa 1995), *Orichophragmus violaceus* (Luo *et al.* 2000), tomato (Dielen *et al.* 2001), and rose (Vu *et al.* 2006). In contrast, no difference in the rate of *in vitro* flowering *Bambusa edulis* plantlets was observed as a result of various concentrations of sucrose and nitrogen in the media (Lin *et al.* 2003).

The NH_4^+/NO_3^- ratio is an important factor for *in vitro* flowering: increasing this ratio depressed *in vitro* flowering whereas reducing the NH_4^+ concentration promoted *in vitro* flowering (Kachonpadungkitti *et al.* 2001). High content of phosphorous in the medium stimulated flowering in *Cymbidium* (Duan and Yazawa 1994, Kostenyuk *et al.* 1999, Tee *et al.* 2008). In the present study, a similar result was obtained when the ratio of KNO_3 to NH_4NO_3 was enhanced about 2- or 3-fold

Table 5. Effect of different plant growth regulators [mg dm^{-3}] on *in vitro* flowering miniature hybrid rose after 60 d of culture on MS basal medium containing 50 g dm^{-3} sucrose. Means ± SE of 600 replicates; different letters within a column indicate significant differences at $P \leq 0.05$ according to DMRT.

TDZ	BA	Zeatin	KT	NAA	IBA	Flowering [%]
0	0	0	0	0	0	0.0 u
0.1	0	0	0	0	0	15.67 ± 1.33 s
0.2	0	0	0	0	0	25.67 ± 1.67 no
0.5	0	0	0	0	0	28.33 ± 1.67 mn
1.0	0	0	0	0	0	34.00 ± 3.21 hij
2.0	0	0	0	0	0	31.33 ± 1.67 jkl
0	0.5	0	0	0	0	22.33 ± 2.20 pqr
0	1.0	0	0	0	0	35.67 ± 1.33 gh
0	2.0	0	0	0	0	41.67 ± 1.33 de
0	3.0	0	0	0	0	45.33 ± 1.88 c
0	4.0	0	0	0	0	31.00 ± 1.58 klm
0	0	0.1	0	0	0	8.00 ± 1.58 t
0	0	0.2	0	0	0	21.67 ± 1.88 qr
0	0	0.5	0	0	0	28.67 ± 1.88 lm
0	0	1.0	0	0	0	38.00 ± 1.58 fg
0	0	2.0	0	0	0	24.33 ± 1.33 opq
0	0	0	0.5	0	0	0 u
0	0	0	1.0	0	0	8.33 ± 1.33 t
0	0	0	3.0	0	0	17.67 ± 1.88 s
0	0	0	5.0	0	0	25.00 ± 1.58 op
0	0	0	7.0	0	0	20.67 ± 2.76 r
1.0	0	0	0	0.1	0	40.33 ± 1.88 ef
1.0	0	0	0	0.2	0	44.33 ± 2.58 cd
1.0	0	0	0	0	0.1	37.67 ± 2.45 fg
1.0	0	0	0	0	0.2	34.67 ± 1.88 hi
0	3.0	0	0	0.1	0	52.33 ± 1.45 a
0	3.0	0	0	0.2	0	49.67 ± 1.88 ab
0	3.0	0	0	0	0.1	48.67 ± 1.67 b
0	3.0	0	0	0	0.2	39.67 ± 1.88 ef
0	0	1.0	0	0.1	0	40.33 ± 2.45 ef
0	0	1.0	0	0.2	0	35.33 ± 1.33 gh
0	0	1.0	0	0	0.1	32.00 ± 2.15 ijk
0	0	1.0	0	0	0.2	40.00 ± 2.00 ef
0	0	0	0	0.2	0	0 u
0	0	0	0	0.5	0	0 u
0	0	0	0	0	0.2	0 u
0	0	0	0	0	0.5	0 u

and the percentage of *in vitro* flowering was also significantly increased when the content of KH_2PO_4 was increased.

BA has been used for most experiments , *Dendrobium primulinum* (Deb and Sungkumlong 2009), × *Doriella* cv. Tiny (Duan and Yazawa 1994), *Dendrobium* cv. Chao Praya Smile (Hee *et al.* 2007), *Lilium rubellum* (Ishimori *et al.* 2009), *Cymbidium niveo-marginatum* (Kostenyuk *et al.* 1999), *Withania somnifera* (Saritha and Naidu 2007), *Dendrobium* cv. Madame Thong-In (Sim *et al.*2007), *Dendrobium candidum* (Wang *et al.* 1997), *Phalaenopsis* (Duan and Yazawa 1995), *Rosa* cultivars (Wang *et al.* 2002; Vu *et al.* 2006), and *Solanum nigrum* (Sridhar and Naidu 2011). Nevertheless, the most suitable BA concentration showed huge differences depending on the plant species.

Table 6. Effect of day/night temperature (12-h photoperiod) on *in vitro* flowering miniature hybrid rose after 60 d of culture on MS basal medium containing 3.0 mg dm^{-3} BA, 0.1 mg dm^{-3} NAA and 50 g dm^{-3} sucrose. Means ± SE of 600 replicates; different letters within a column indicate significant differences at $P \leq 0.05$ according to DMRT.

Temperature [°C]	Flowering [%]
32/32	44.33 ± 2.58 c
30/30	50.00 ± 2.00 b
28/28	52.33 ± 1.45 b
26/26	48.67 ± 1..67 b
24/24	42.00 ± 1.58 c
28/20	65.67 ± 2.08 a

Kostenyuk *et al.* (1999) reported that 2.5 mg dm^{-3} TDZ was more effective than BA for flower bud induction in *Cymbidium niveo-marginatum*, although TDZ resulted in poor plant growth, whereas floral buds withered rapidly. The positive effect of TDZ was also observed in studies of *in vitro* flowering bamboo (Lin *et al.* 2003, 2004). The most efficient flower bud induction (49.1 and 44.1 %) was possible on media supplemented with 0.5 mg dm^{-3} TDZ and 0.1 mg dm^{-3} NAA, or 0.5 mg dm^{-3} ZT and 0.1 mg dm^{-3} NAA for rose cv. Orange Parade with TDZ and ZT being the best choice for flower induction in all six cultivars tested (Wang *et al.* 2002). However, ZT was also reported to inhibit floral bud formation in *Torenia* (Tanimoto *et al.* 1981). Wang *et al.* (2002) also reported that higher concentrations of TDZ (1.0 or 2.0 mg dm^{-3}) resulted in a lower flower bud induction (25.8 and 9.2 %, respectively). In the present study, 3.0 mg dm^{-3} BA was more suitable for flowering *in vitro* than TDZ, ZT, and KIN.

When only NAA or IBA were used, all buds formed roots but did not develop flowers (Table 5). Goh and Yang (1978) also demonstrated that IAA can suppress the promotive effect of BA on *in vitro* flowering in two *Dendrobium* hybrids. Wang *et al.* (1997) reported that there were a few *Dendrobium candidum* plantlets with roots that flowered *in vitro* but the percentage of *in vitro* flowering was much lower than of rootless plantlets. Wang *et al.* (1997) also reported that NAA alone suppressed flower formation. The cause may be that roots act as the major site of cytokinin biosynthesis, thus plantlets with roots were less sensitive to exogenous cytokinin than rootless ones (Wang *et al.* 1997); on the other hand, the regenerative roots may be a sink for some substances that are required for floral initiation or for the release of some signals which inhibit flower induction, as occurs in tobacco (reviewed by McDaniel 1996). In addition, Saxena *et al*. (2008) reported that the frequency of flowering *Vigna aconitifolia* approached 100 % when 3.78 or 11.34 mg dm^{-3} ABA and 92 g dm^{-3} proline were

added to medium. ABA is considered to be a stress hormone and functions through a set of ABA-regulated genes which in turn lead to accumulation of osmo-protectants like proline.

In general, a low-temperature requirement for *in vivo* floral induction has been documented in *Phalaenopsis* (Sakanishi *et al.* 1980). Gibberellins and temperature influenced sugar content and so flowering in *Phalaenopsis* (Chen *et al.* 1994) as sugars are signals that could take part in growth and morphogenesis (Bernier *et al.* 1993). However, Sakanishi *et al.* (1980) reported that low temperature treatments (using day/night temperatures of 25/15 and 25/10 °C) did not promote the formation of floral buds and suggested that the conditions for the induction of floral buds in *Phalaenopsis in vitro* might be different from those *in vivo* (Duan and Yazawa 1995). Vaz *et al.* (2004) reported that an orchid *Psygmorchis pusilla* maintained at 24 or 30 °C had similar flowering responses, 27 °C being the most adequate; in addition, within the temperature range of 24 to 30 °C, the higher the temperature, the earlier flower buds wilting occurred. High temperature increased respiration rate and lowered CO_2 absorption resulting in sugar depletion, thereby inhibiting growth and delaying flowering. Wang *et al.* (2009) reported deformed flower development at 25 °C, but normal development at a lower temperature regime (23/18 °C) for 45 d. In the present study, the temperature difference between day and night favoured the accumulation of sugars as it was reported by Bernier *et al.* (1993).

The ABC model (and subsequent ABCE model), which explains floral pattern formation, was conceived in the early 1990s and modified over the course of the last two decades based on a series of celebrated homeotic mutants (Coen and Meyerowitz 1991, Pelaz *et al.* 2000, and reviewed by Causier *et al.* 2010). Flower colours are the result of a combination of factors, mainly phyto-chromes (flavonoids, carotenoids, and betalains), vacuolar pH, phytohormones, and growth stages (Giuliano *et al.* 1993, Weiss *et al.* 1995, Tanaka *et al.* 1998). The diverse flower colours observed in nature are caused by different modifications of anthocyanidins (flavonoids), such as methylation, glycosylation, and acylation (reviewed by Yonekura-Sakakibara *et al.* 2009). These flowering models could serve as a basis for explaining some of the flower mutants observed in this study.

The key regulatory genes for flowering and morpho-logy in plant species have diverged and evolved to uniquely adapt to different environmental conditions. The plant phosphatidyl ethanolamine-binding protein-like genes *TERMINAL FLOWER1* (*TFL1*)-like and *CENTRORADIALIS* (*CEN*)-like which were involved in the control of shoot meristem identity and flowering time, were isolated and compared (Mimida *et al.* 2012). This study successfully established a system for induction of *in vitro* flowering rose. In that process, some different types of incomplete or abnormal floral structures were observed to form in *in vitro* flowers (Fig. 1*G-N*). Similar abnormalities were observed previously in other plant species during *in vitro* flowering (Duan and Yazawa 1994, Chia *et al.* 1999, Saritha and Naidu 2007). The various abnormalities observed in *in vitro* flowers indicate that different conditions might be required for the initiation and development of flowers (Wang *et al.* 1993). Although the characteristics of these abnormal flowers could not be stably inherited, these phenomena and materials have the potential to make *in vitro Rosa* flowering an ideal experimental system for research on the physiological and molecular mechanisms of rose flowering which have to date not yet been explored.

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