ELSEVIER

Scientia Horticulturae 63 (1995) 47–55

SCIENTIA HORTICULTURÆ

Development of axillary buds of rose in vitro

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Accepted 3 February 1995

Abstract

An in vitro model system has been developed to grow axillary buds into shoots, which are morphologically comparable to those grown in vivo. In the development of the shoot three stages can be distinguished: sprouting of the bud, unfolding of leaves already present in the bud, and new formation of leaves and a flower bud. A low concentration of benzyladenine (BA), sugar (preferably glucose), and a cultivar-dependent concentration of a suitable agar was necessary to obtain a complete shoot. The size of the in vitro shoot positively correlated with the size of the explant. The presence of the petiole inhibited the outgrowth of lateral buds, which were already present in the inoculated bud. Using this in vitro system the growth potential of axillary buds, apart from influences of other plant parts, can be studied.

Keywords: Axillary bud; Micropropagation; Model system; Morphology; *Rosa hybrida*

1. Introduction

The flower yield of roses depends on the willingness of axillary buds to sprout and their subsequent growth into flowering shoots (Zieslin et al., 1973). The development of axillary buds on intact plants depends on three partly interdependent factors, i.e. the intrinsic growth potential of the buds, their position on the plant (Zieslin et al., 1976) and influences of other plant parts (Zieslin and Halevy, 1976). This latter point, especially, makes it difficult to assess the growth potential of the buds themselves in intact plants. In vitro culture proved useful to study bud and shoot growth, isolated from the interactions to which the buds are

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Abbreviations: BA = Benzyladenine; GA = Gibberellic acid; IBA = Indole butyric acid; IPA = Indole propionic acid; 2iP = 2-isopentenyladenosine; MS = Murashige and Skoog; NAA = Naphthaleneacetic acid.

SSDI 0304-4238(95)00786-5

subjected in the intact plant. Moreover, it offers a technique to study the physiology and requirements for growth and development of isolated organs (Dutcher and Powell, 1972; Halim et al., 1988; Nadel et al., 1991).

In each leaf axil of a rose shoot an axillary bud is present. A quiescent axillary bud of a flowering shoot contains the lower part of the future shoot, i.e. six to seven scale-like leaves and four to five compound leaves. When the correlative inhibition of the axillary bud is released by removing the stem above the bud, the bud will sprout and approximately seven new leaves and a flower will be formed (Marcelis-van Acker, 1994).

In vitro culture of rose has been described previously, but has focused on micropropagation (Jacobs et al., 1969; Hasegawa, 1979; Bressan et al., 1982), somatic embryogenesis and adventitious shoot formation (Tweddle et al., 1984). The studies on in vitro culture of rose have been reviewed by Short and Roberts (1991). The methods of micropropagation of rose are always based on the system of multiple shoot formation, starting with axillary buds or shoot tips, which are regularly subcultured. In most cases a standard Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) is used with a rather high benzyladenine (BA) concentration. The shoot proliferation may decline after a number of subcultures at a high BA concentration, as reported by Norton and Norton (1986). Furthermore, in vitro derived plants often show more branching after hardening than in vivo plants (Dubois et al., 1988; Vijaya and Satyanarayana, 1991), which might be a carry-over effect of the relatively high BA concentration applied in the multiple shoot system. The multiple shoot system is not a suitable system to study the growth potential of axillary buds in vitro.

The aim of the present study was to develop an in vitro model system for physiological studies on the development of axillary rose buds. In this system axillary shoots of rose are grown as single elongated shoots at low cytokinin concentration. The effects of medium components (agar, plant growth regulators and sugars), petiole and explant size on bud and shoot development were evaluated. Furthermore, the growth characteristics of the in vitro shoot were compared with those of the in vivo shoot.

2. Materials and methods

From two cultivars of *Rosa hybrida*, 'Sweet Promise' and 'Motrea', stem segments about 1 cm long, bearing a quiescent axillary bud including a petiole stump of 1 cm were cut from the middle part of flower stems in the harvestable stage (sepals reflexing). Flower stems were used immediately after harvest. The explants were surface-sterilised in 70% alcohol for a few seconds, followed by 20 min in 1% NaOCl. Explants were then washed three times with sterile water. Before inoculation the buds were excised, leaving as few stem and petiole tissue as possible. The effect of explant size was investigated by varying the stem length (0, 0.5 and 2.0 cm) or by varying the petiole length (0, 0.5, 1.0, 1.5 and 2.0 cm).

The basic culture medium consisted of MS salts with 37.5 mg l^{-1} NaFeEDTA. Unless stated otherwise, 45 g l^{-1} glucose, 0.1 mg l^{-1} BA and 5 g l^{-1} agar (MC 29; Lab M, Bury, UK) were used. Preliminary results showed that, in contrast to 'Sweet Promise', 'Motrea' required the addition of MS vitamins and glycine. The effect of sugar type was evaluated by comparing sucrose with glucose at a concentration of 45 g l^{-1}. The effect of BA concentration was investigated by applying 0, 0.044, 0.22, 0.44, 0.88 and 2.2 μM BA. The

effect of cytokinin type was studied by comparing equimolar concentrations (0.44 μM) of the cytokinins BA, zeatin, zeatin riboside, 2-isopentenyladenosine (2iP), indole propionic acid (IPA) and kinetin. The effect of agar brand was investigated by testing six different commercially available agars at a concentration of 5 g l^{-1}, i.e. Daichin (Brunschwig Chemie, Amsterdam, Netherlands), Difco Bacto (Difco, Detroit, USA), MC 29 (Lab M, Bury, UK), BD (Becton Dickinson, Cockeysville, USA) grade A, BD granulated, and BD purified. Furthermore, for agar MC 29 a concentration range (5, 6, 7 and 8 g l^{-1}) was applied. The pH was adjusted to 5.8. Culture tubes were filled with 15 ml medium, closed with a cotton plug, and autoclaved for 20 min. Tubes with an explant were sealed with Vitafilm (Good Year) and incubated in a culture room at 23°C with a 16 h photoperiod (5–7 W m^{-2}, Philips TL 54).

Routinely, the effects of the treatments on growth and development of the axillary buds into shoots were evaluated after 4 weeks by determining length, weight and number of compound leaves of the main shoot, and number of lateral shoots. The experiment on cytokinin type was evaluated after 5 weeks. For calculation of the means only sprouted buds were taken into account.

The effects of medium components and explant size were investigated in one or two replicate experiments. Per treatment 12, 18, or 24 replicate explants were used. When one experiment was performed the mean and the standard error of the mean (SE) were calculated per treatment. In the case of two experiments analysis of variance was applied and the significance of differences was determined by Student's t-test ($P = 0.05$).

3. Results

3.1. Morphology

In general, at least 90% of the axillary buds inoculated in vitro sprouted (length over 0.5 cm; stage I). On most media, the four to five leaves and internodes which were already in the bud at the time of inoculation, unfolded (stage II). When cultured under optimal conditions, approximately seven additional leaves and internodes and a flower were formed (stage III). Comparative studies revealed that this developmental process under optimal conditions in vitro is very similar to that in vivo, resulting in a miniature version of the in vivo plant. The number of leaves preceding the flower and the leaf form (number of leaflets per leaf) of in vitro grown shoots were very similar to those of in vivo grown shoots.

3.2. Medium components

3.2.1. Plant growth regulators

For both cultivars the presence of a cytokinin was necessary for prolonged shoot growth of axillary buds; without cytokinin, axillary buds did sprout, but hardly developed further than stage II, since only a few leaves were formed and little shoot elongation occurred (Table 1). A concentration of 0.44 μM BA resulted in elongated shoots with a flower bud, whereas higher concentrations induced a cluster of shoots. At high concentrations weight and length of the main shoot decreased (Table 1). Comparing equimolar concentrations

Table 1
Effect of BA concentration on axillary shoot development from single node explants of rose 'Sweet Promise'. Values are the mean of 48 plants

BA conc. (μM)	No. of leaves	Length (cm)	Weight of main shoot (g)	No. of laterals	Total weight (g)
0	6.0	1.0	0.24	0.1	0.24
0.044	6.9	1.2	0.23	0.1	0.23
0.22	10.1	2.3	0.25	0.5	0.25
0.44	11.3	3.4	0.30	1.2	0.34
0.88	11.6	3.0	0.28	2.3	0.37
2.2	11.5	1.7	0.24	2.7	0.46
LSD ($P=0.05$)	0.9	0.6	0.06	0.9	0.08

(0.44 μM) of the cytokinins BA, zeatin, zeatin riboside, 2iP and IPA showed that all cytokinins, except kinetin, stimulated the growth of axillary buds into shoots of 'Sweet Promise' (Fig. 1). BA, zeatin and zeatin riboside resulted in the longest shoots with the highest weight (Fig. 1). Increasing the concentration of kinetin to 9.3 μM had no effect on weight, length and number of leaves of 'Motrea' (data not shown).

3.2.2. Agar

Both cultivars appeared to be sensitive to the agar brand. In several experiments the agars Daichin (agar 1), MC 29 (agar 3), and BD purified (agar 6) gave the best results. In Fig. 2 a representative experiment is shown. Since MC 29 showed least symptoms of necrosis of the apex, this agar was routinely used.

With respect to the optimal agar concentration large differences between the cultivars were observed. 'Motrea' preferred high concentrations of agar. At 7 g l^{-1} completely developed shoots were formed (Fig. 3). 'Sweet Promise', however, showed the best results

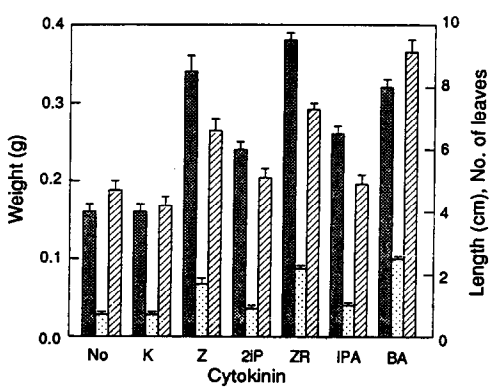

Fig. 1. Effect of cytokinin type (0.44 μM) on number of leaves (hatched bars), length (dotted bars) and weight (cross-hatched bars) of axillary shoots from single node explants of rose 'Sweet Promise'. Values are the mean of 18 explants. Bars indicate SE. No, no cytokinin added; K, kinetin; Z, zeatin; ZR, zeatin riboside.

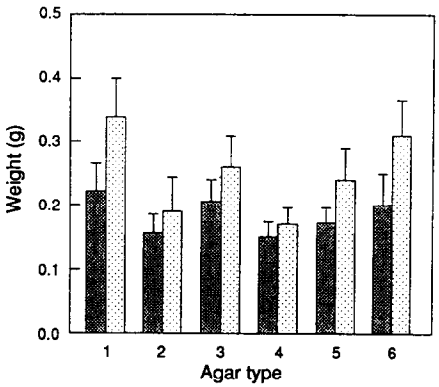

Fig. 2. Effect of the agar brand on weight of axillary shoots from single node explants of rose 'Motrea' (cross-hatched bars) and 'Sweet Promise' (dotted bars). Values are the mean of 12 plants. Bars indicate SE. Agar 1, Daichin; agar 2, Difco Bacto; agar 3, MC 29; agar 4, BD grade A; agar 5, BD granulated; agar 6, BD purified.

with extremely low concentrations (4 g l^{-1}) or when grown on small rockwool plugs with liquid medium (data not shown).

3.2.3. Sugar

For both cultivars glucose gave better growth than sucrose (Table 2). For 'Motrea', development of the buds was more enhanced with glucose in the medium. The time of addition of 45 g l^{-1} sucrose, before or after autoclaving the medium, did not make any difference. Chemical analysis with HPLC showed that after autoclaving only 3% of the sucrose was decomposed while glucose could be recovered completely.

3.3. Explant factors

Shoot growth increased with increasing stem tissue. No effect on developmental stage was found since the number of leaves was similar (Table 3). Absence of the petiole induced

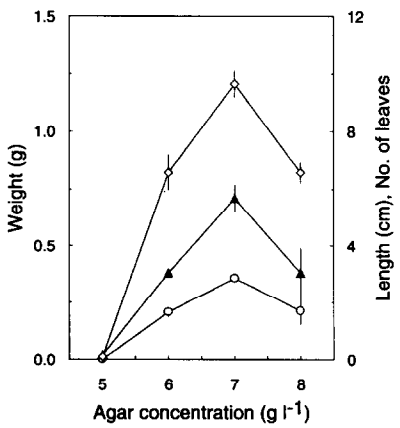

Fig. 3. Effect of agar (MC 29) concentration on weight (▲), length (○) and number of leaves (◇) of axillary shoots from single node explants of rose 'Motrea'. Values are the mean of 12 plants. Bars indicate SE.

Table 2
Effect of sugar type at 45 g l^{-1} on axillary shoot development from single node explants of rose 'Sweet Promise' and 'Motrea'. Values are the mean of 48 plants

Cultivar	Sugar	No. of leaves	Length (cm)	No. of laterals	Total weight (g)
'Sweet Promise'	Sucrose	10.3	2.5	1.6	0.25
	Glucose	10.8	3.4	1.6	0.33
LSD ($P=0.05$)		1.3	0.4	0.7	0.03
'Motrea'	Sucrose	8.9	0.5	0	0.10
	Glucose	14.0	1.9	0	0.27
LSD ($P=0.05$)		1.9	0.6	–	0.08

Table 3
Effect of explant size on axillary shoot development from single node explants of rose 'Sweet Promise'. Values are the mean of 48 plants

Explant	No. of leaves	Length (cm)	No. of laterals	Total weight (g)
Axillary bud	8.8	1.7	0.9	0.22
Stem slice of 0.5 cm	8.3	2.0	0.6	0.26
Stem slice of 2.0 cm	8.4	2.5	0.7	0.44
LSD ($P=0.05$)	0.7	0.4	0.6	0.02

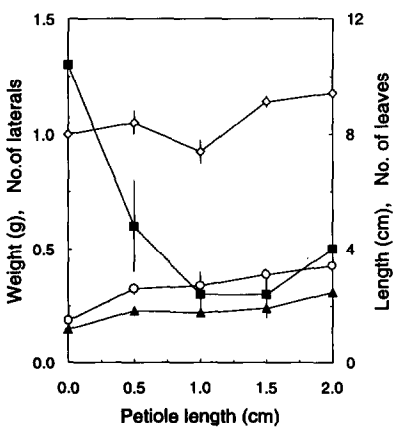

Fig. 4. Effect of petiole length on weight (▲), number of laterals (■), length (○) and number of leaves (◇) of axillary shoots from single node explants of rose 'Sweet Promise'. Values are the mean of 24 plants. Bars indicate SE.

growth of lateral shoots, whereas the main shoot remained short (Fig. 4). The pattern of the effect of BA concentration on explants was not influenced by the presence of the petiole, but the optimum concentration was lower in its absence (0.22 μM instead of standard 0.44 μM). An increase in petiole length resulted in a slight increase in both shoot length and shoot weight, while the numbers of leaves and laterals were not substantially affected (Fig. 4).

4. Discussion

Axillary buds are not dormant but correlatively inhibited by upper plant parts (Zieslin and Halevy, 1976). As soon as the inhibition is released in vivo, by pruning the stem part above the bud, the bud will sprout and develop into a shoot. The medium requirements for bud break and unfolding of preformed stem parts in vitro appeared not very specific. As the sprouting bud is a strong sink for assimilates (Mor and Halevy, 1979), carbohydrates were supplied to the bud by addition of sugar to the medium. The effect of different sugars on growth of rose in vitro has not been reported before. Commonly sucrose is supplied, only Ghashghaie et al. (1992) used glucose. The present study shows that glucose induces more vigorous growth and especially for 'Motrea', use of glucose should be strongly advised.

In vivo, the apical meristem completes its developmental programme, after release from correlative inhibition, by forming several additional leaves and a terminal flower bud. In vitro, the composition of the medium determines which developmental stage is reached. Addition of cytokinin to the culture medium appeared necessary for the bud to complete its developmental programme isolated from the intact plant. Accordingly, axillary buds of pea did not form new leaves and grew only slightly on a medium without cytokinin (Gould et al., 1987). Excised floral buds are also reported to require cytokinin for the growth and development of floral organs (Rastogi and Sawhney, 1989). In vivo, cytokinin will primarily be supplied by the roots, that are absent in vitro. Of the several cytokinins tested, BA appeared most suitable, although it should be noted that the optimum concentration might be dependent on the cytokinin used. The BA concentration applied in the multiple shoot system is supra optimal for the single shoot system, since also the buds, which are already present in the axils of the bud scales (Marcelis-van Acker, 1994), were released from inhibition, resulting in a cluster of shoots. Outgrowth of the buds was accompanied by a smaller main shoot (Table 1), indicating that the main shoot experienced competition of the axillary shoots.

The agar brand may affect the growth and development of in vitro plants (Debergh, 1983). Rose also appeared to be very sensitive to agar brand. Several explanations have been given for the agar effect on in vitro cultures, e.g. availability of water or nutrients (Debergh, 1983; Bornman and Vogelmann, 1984; Scherer et al., 1988; Ghashghaie et al., 1991). In case of rose the nutrient availability may be a likely explanation. For, a reduced nutrient supply at higher agar concentration agrees very well with the observation that 'Sweet Promise', in contrast to 'Motrea', is not able to grow at lower nutrient concentration than the standard MS medium (H.J. Scholten, unpublished data, 1994). Moreover, addition of liquid medium to the agar medium after 3 weeks of culture (Maene and Debergh, 1985) had a positive effect on growth of 'Sweet Promise'.

The effect of explant size on growth of the axillary bud might be nutritional and/or hormonal. The data on the effect of stem tissue attached to the excised axillary bud (Table 3) suggest a nutritional effect of the stem tissue. The effect of the petiole might also be hormonal, since absence of the petiole induced release of the buds in the axils of the bud scales of the inoculated bud.

The single shoot system is suitable for physiological studies on axillary bud development. By use of this system the effects of influencing factors imposed during initiation and early development of the axillary bud on its subsequent growth potential can be studied and quantified. This knowlegde will lead to a better understanding of axillary bud development and of the growth of a rose plant. Furthermore, the single shoot system offers possibilities for micropropagation by single-node culture. In this way, carry-over effects after transfer to in vivo conditions, resulting from the high cytokinin concentration in the culture medium, may be reduced.

Acknowledgements

We would like to thank Prof. Dr. R.L.M. Pierik and Prof. Dr. J. Tromp for valuable comments on the manuscript.

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