

***In vitro* Conversion of Alginate-encapsulated Nodal Segments of *Rosa hybrida* L. 'Kardinal' Into Complete Plantlets**

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Abstract

The present experiments were conducted at Humboldt University of Berlin, Horticultural Plant Systems, Germany during 2012 and 2013 seasons, to clarify the nutrient requirements for regeneration of encapsulated nodal segments of *Rosa hybrida* 'Kardinal', as well as define the nutrient losses from the beads during incubation on nutrient-free media. Shoot cultures were cultivated on modified MS medium. For encapsulation, 4 to 5 mm nodal segments were isolated 3 weeks after subculturing. The nodal segments were put in 3% Na-alginate diluted in the modified MS medium with various sucrose concentrations (30 to 90 g l⁻¹) and 0.5 to 2 mg l⁻¹ indole-3-butyric acid (IBA). To improve the nutrient supply, 85 µl, 95 µl or 105 µl of Na-alginate with one nodal segment was dropped in to 75 mM CaCl₂ dissolved in the same media contains Na-alginate for 30 min hardening. The results showed that already after 24h incubation on the water-agar-medium more than 90% of nitrate was diffused out of the beads. The reduction of water-agar-medium volume from 25 ml to 7 ml reduced the nutrient leakage and improved the shoot formation from 40 to 90%. The root regeneration of the encapsulated nodal segments also increased from 40% to 80% either by increasing the bead volume from 85 to 105 µl or the sucrose concentration from 30 to 50 g l⁻¹ in the propagation medium. The highest conversion (approximately 50%) was found after supplying 1 mg l⁻¹ IBA and 90 g l⁻¹ sucrose to the bead gel matrix.

Keywords: *Rosa hybrida*, artificial seeds, gel matrix compounds, bead volume, nutrient leakage

Introduction

Creation of 'artificial seeds' by encapsulation of somatic embryos, shoot tips and buds has been under discussion since the first publication of this idea by Murashige (1977). Using nodal segments or shoot tips for encapsulation has some advantages in comparison with somatic embryos. First, a higher genetic stability of plant material of the nodal segments or shoot tips can be expected (Lata *et al.*, 2011, Standardi and Micheli, 2012, Sharma *et al.*, 2013 and

Gholami and Kaviani, 2018). This seems to be very important for plants with a high mutability like *Chrysanthemum* (Teixeira da Silva, 2004) and also *Rosa spp.* in which somaclonal variation was found after callus culture (Rout *et al.*, 1999). Second, the non-embryogenic vegetative propagules can be used for mass clonal propagation of high value crops at a reasonable cost especially when somatic embryos are not available for encapsulation (Maruyama *et al.*, 1997, Chand and Singh, 2004,

Rai *et al.*, 2008 and Elangomathavan *et al.*, 2017). In the unipolar nodal segments not only growing processes like those in the bipolar somatic embryos have to be started, but also the energy consuming and oxygen required regeneration process of adventitious roots has to be initiated and completed (Standardi and Piccioni, 1997 and Calamar and De Klerk, 2002). Up to now, encapsulation of shoot segments with Ca-alginate has been published for various species (Singh *et al.*, 2010, Carlson and West, 2016, Gantait *et al.*, 2017 and Sharma and Sharma, 2018).

Most protocols used a modified medium after Murashige and Skoog (1962) as growing medium guarantee the nutrient supply for the encapsulated plant material over a long period of time. If growing media was used without nutrient supply, the growing response of the encapsulated plant material became low or sometimes no conversion could be occurred (Pattniak and Chand, 2000, Pinker and Abdel-Rahman, 2005 and Pintos *et al.*, 2008). The germination of artificial seeds directly on substrates under non-sterile conditions requires sufficient nutrients to be supplied within the bead as an artificial endosperm (Pinker and Abdel-Rahman, 2005 and Benelli *et al.*, 2017). Taha *et al.* (2013) compared synthetic seeds of *Oxalis triangularis* sown directly on MS basal medium, black soil and sterile black soil. They showed that conversion frequency of synthetic seeds into complete plantlets was better under *in vitro* than *in vivo* conditions.

Recently, for encapsulation, mostly Ca-alginate is used for its gel-

ling properties and the suitability for plant material (Saiprasad, 2001, Islam and Bar, 2012 and Rihan *et al.*, 2017). The beads formed after dropping the Na-alginate in CaCl₂ solution (75 to 100 mM) are wet, sticky and not protected against water loss by evaporation. Moreover, it has to be expected that also bead gel matrix compounds like nutrients and growth regulators diffuse from the bead to the substrate till the chemical equilibrium is reached. So far, there are only few data available about the extent and the time course of this nutrient leakage of the beads (Redenbaugh *et al.*, 1987 and Timbert *et al.*, 1997).

The current experiments aimed at optimising the conversion of encapsulated nodal segments of *Rosa hybrida* 'Kardinal' (a tea rose cultivar used for cut flowers) cultured on a nutrient-free medium and prepared for *ex vitro* conversion into whole plantlets.

Material and Methods

The present experiments were conducted at Humboldt University of Berlin, Horticultural Plant Systems, Berlin, Germany during 2012 and 2013 seasons, aiming at getting more information about the nutrient requirements for growing and proliferation of encapsulated nodal segments of *Rosa hybrida* 'Kardinal', as well as the nutrient losses from the beads during incubation on nutrient-free media. Shoot cultures of *Rosa hybrida* 'Kardinal' were cultured on modified MS basal salt medium (Murashige and Skoog, 1962), and subcultured at 6-week-intervals. The medium supplemented with 2.5 mg l⁻¹ thiamin-HCl, 0.2 mg l⁻¹ pyridoxin-HCl, 0.2 mg l⁻¹ biotin, 100 mg l⁻¹

myo-inositol, 0.25 mg l⁻¹ 6-benzylaminopurine (BAP) and 0.5 mg l⁻¹ gibberellic acid (GA₃), 8 g l⁻¹ agar (Serva Kobe I), and 30 g l⁻¹ sucrose. For a special pre-treatment of shoot cultures, the sucrose content was increased (35, 40, 45, and 50 g l⁻¹). The pH was adjusted at 5.8 before autoclaving at 121°C under 1.5 Kg/cm² for 15 min. Cultures were maintained at 24±1°C and illuminated 16h daily with 35 µmol m⁻² s⁻¹ photosynthetically active radiation (white light, Osram L58 W/30).

For encapsulation, 4 to 5 mm long nodal segments were isolated 3 weeks after subculturing. The nodal segments were put in 3% Na-alginate (Fluka) diluted in the modified MS medium with various sucrose concentrations (30, 40, 50, 60, 70, 80 and 90 g l⁻¹). The plant growth regulator was 3-indolebutyric acid (IBA) at 0.5, 1 or 2 mg l⁻¹. Droplet of 85, 95 and 105 µl containing one segment each put into 75 mM CaCl₂ solution dissolved in the same modified MS medium and slowly agitated for 30 min. Thirty beads were used per treatment distributed to three replicates in 100 ml Erlenmeyer flasks. Each flask contained 25 ml water-agar-medium solidified with 8 g l⁻¹ agar (Serva Kobe I) at pH 5.8 and was covered with aluminium foil. In the 1st experiment, the volume of water-agar-medium was reduced from 25 ml to 12 and 7 ml to improve the nutrient supply. The second experiment was carried out to investigate the effect of sucrose concentrations (30, 35, 40, 45 and 50g l⁻¹) in the pre-treatment propagation medium on shoot development and root regeneration of encapsulated nodal segments of *R. hybrida* `Kardi-

nal' on a water-agar-medium. Details for gel matrix compounds of the beads and water-agar-medium are indicated in the result section. The percentages of shoot development and root regeneration of rose encapsulated nodal segments were recorded after 7 weeks.

To investigate the nutrient losses from the beads to the growing medium, beads were used with a volume of 85 µl without plant segments. Ten beads were incubated per Erlenmeyer flask with 7, 12 and 25 ml water-agar-medium. After 1 and 7 days on a water-agar-medium, 30 beads were incubated for 3h in 100 ml deionised water to leach all nutrients from the beads. The nitrate content of this solution was determined with a nitrate selective electrode (NO 500, Ingold) linked with the Ionometer (WTW 2000 mx).

All experiments were conducted 3 times. Data were evaluated with Chi-square-test (Pearson, P≤ 0.05) for statistical significance.

Result and Discussion

Effect of the bead gel matrix components

The first experiment was conducted to investigate the effect of IBA concentrations in the bead gel matrix on rooting of rose encapsulated nodal segments on a water-agar-medium (Fig. 1). Results showed that both shoot and root formation were significantly affected by IBA concentrations. At lower IBA concentrations (0.5 and 1 mg l⁻¹), ca. 50 to 53.9% of the encapsulated nodal segments developed shoots but only 8 to 18% regenerated roots. Increasing IBA concentration from 1 to 2 mg l⁻¹ significantly increased root regeneration

from 18.0 to 48.0%, but inhibited shoot development from 53.9 to 23.0% (Fig. 1). These results are quite different from that of *Dendranthema x grandiflora* with up to 100% conversion reported earlier in similar culture conditions (Pinker and Abdel-Rahman, 2005), which indicates difficulties in stimulating the conversion of rose encapsulated nodal segments. However, similar results were reported in other rose species. The shoot development was quicker and easier than root regeneration in encapsulated axillary buds of *Chamaecyparis obtusa* (Okamura *et al.*, 1994) and mulberries (Pattniak and Chand, 2000). Standardi and Piccioni (1997) pointed out that rooting was always

the limiting factor for conversion of encapsulated buds of apple rootstock. Piccioni (1997) suggested an auxin pulse treatment for 24h and preculturing the treated nodal segments of apple rootstock for 3 to 9 days before encapsulation. Conversion of encapsulated shoot tips and nodal segments of eucalypt, *Corymbia torelliana* x *C. citriodora* into plantlets was achieved on half-strength MS medium by treating shoot tips or nodes with 4.9-78.4 μM IBA prior to encapsulation. Pre-treatment with 19.6 μM IBA provided 62-100% conversion, and 95-100% of plantlets survived after acclimatization under nursery conditions (Hung and Trueman, 2012).

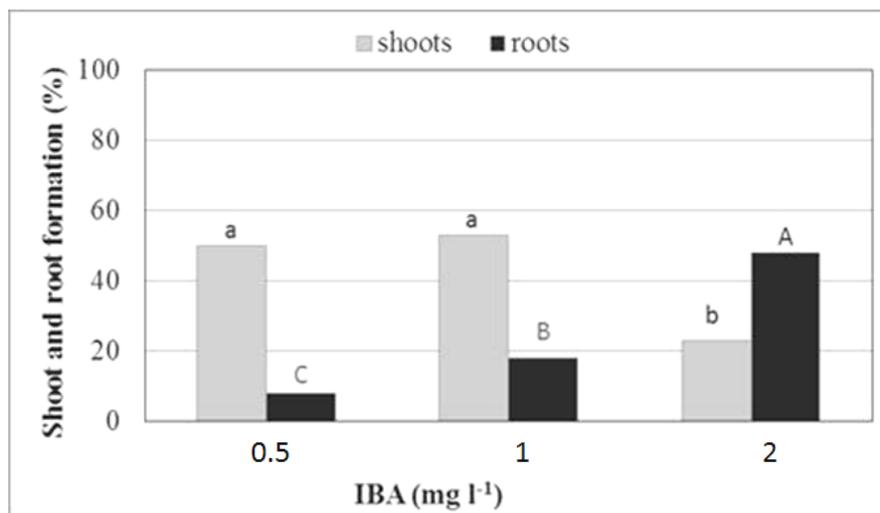


Fig. 1. Effect of IBA concentration in the bead gel matrix (MS + 30 g l⁻¹ sucrose) on shoot and root development of encapsulated nodal segments of *R. hybrida* 'Kardinal' on a water-agar-medium after 7 weeks. Different letters indicate significant differences (Chi square test, Pearson, P<0.05).

However, the next experiment showed that the stimulation of shoot and root development in *R. hybrida* 'Kardinal' was also significantly dependent on the sucrose content of the beads (Fig. 2). The sucrose concentration in the bead gel matrix (MS + 1

mg l⁻¹ IBA) had to be increased even to 80 g l⁻¹ to get ca. 80% shoot development and more than 50% root regeneration on a water-agar-medium. Redenbaugh *et al.* (1987) reported that sucrose represents a source of energy during the shoot proliferation

and can speed up the regrowth of encapsulated propagules. The addition of sucrose into the artificial endosperm enhanced the germination rate of encapsulated somatic embryos in *Camellia japonica* (Janeiro *et al.*, 1997) and *Solanum melongena* (Huda *et al.*, 2007). Sucrose was also the principal determinant for the shoot development of encapsulated buds in *Betula platyphylla* (Kinoshita and Saito, 1990). In addition, Pierik (1997) investigated the effect of macronutrient and sucrose concentration on root formation on stem explants of *Rosa hybrida* 'Montrea'. He reported that macronutrient concentration of full strength MS was optimal for rooting response and the su-

crose concentration should be 45 to 85 g l⁻¹. Rahman *et al.* (2002) recorded better rooting in rose with 40 g l⁻¹ sucrose than with 20 or 60 g l⁻¹. These results agree with our findings (Fig. 2) that sprouting and rooting of rose encapsulated nodal segments requires higher sugar concentrations. Calamar and De Klerk (2002) found that sucrose was essential for apple rooting especially during the first hours on rooting medium. They assumed that in microcuttings, sucrose had a direct effect on rooting by influencing the competence of cells responses to auxin as an energy source during the initial phase of regeneration.

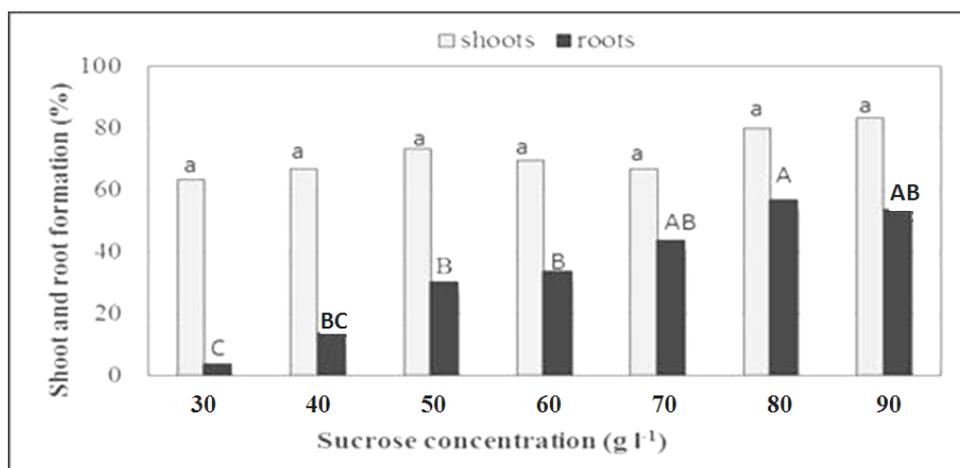


Fig. 2. Effect of sucrose concentration in the bead gel matrix (MS + 1 mg l⁻¹ IBA) on development of encapsulated nodal segments of *R. hybrida* 'Kardinal' on a water-agar-medium after 7 weeks. Different letters indicate significant differences (Chi square test, Pearson, P<0.05).

On the other hand, the present study showed that in beads supplied with 2 mg l⁻¹ IBA, roots were regenerated before shoot development (Fig. 3), while in beads supplied with 1 mg l⁻¹ IBA, the shoots and roots were formed at the same time (Fig. 4). However, the root formation is con-

sidered to be a limiting step for conversion of artificial seeds into plantlets (Standardi and Piccioni, 1997). Therefore, 2 mg l⁻¹ IBA was applied to the beads with *R. hybrida* 'Kardinal' nodal segments in the following experiments.

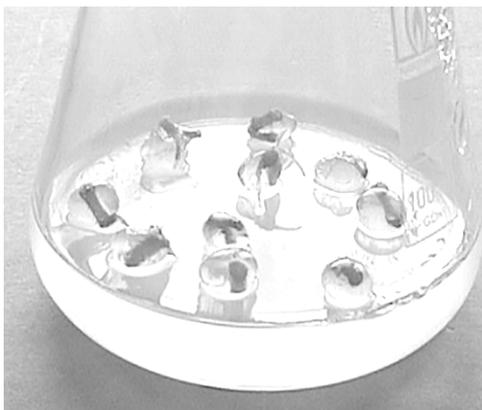


Fig. 3. Root formation of encapsulated nodal segments of *R. hybrida* 'Kardinal' after 2 weeks on a water-agar-medium and MS with 30 g l⁻¹ sucrose and 2 mg l⁻¹ IBA in the bead gel matrix.



Fig. 4. Development of encapsulated nodal segments of *R. hybrida* 'Kardinal' after 7 weeks on a water-agar-medium and MS with 90 g l⁻¹ sucrose and 1 mg l⁻¹ IBA in the bead gel matrix.

Factors affecting the nutrient content within the beads

Following the time course of nitrate leakage (Table 1), it is obvious that already after 24h incubation on the water-agar-medium more than 90% of nitrate was diffused out of the beads by using the standard volume of the water-agar-medium (25 ml). That means after one day, the concentration of nutrients (based on measurement of nitrate content) in the beads is not more than a tenth of the initially added ones. Presumably, this concentration was enough to start growth and regeneration processes in *Dendranthema x grandiflora* encapsulated nodal segments (Pinker and Abdel-Rahman, 2005). However, in *R. hybrida* 'Kardinal' encapsulated nodal segments with low levels of inorganic and organic compounds resulted in very low root regeneration (Fig. 2) as reported earlier by Pierik (1997).

Some increments in the nutrient supply could be gained by reducing the volume of water-agar-medium from 25 to 12 or 7 ml (Table 1). This little change resulted in the best shoot development achieving an increase from 40 to 90% and little improvement of root regeneration recorded an increase from 9 to 25% of encapsulated nodal segments of *R. hybrida* 'Kardinal' (Fig. 5). Timbert *et al.* (1997) determined diffusion of sucrose from the growing medium into the bead and vice versa, and found in alginate beads similar coefficients for both processes. Therefore, we can expect that if the nutrient concentration in the gel matrix is reduced due to the nutrient uptake by nodal segments, nutrients from the growing medium might diffuse back into the bead.

Table 1. Nitrate leakage of beads after incubation on a water-agar-medium with different volumes for up to one week.

Incubation period (Day)	Nitrate loss (%)			
	Medium volume (ml)			
	7	12	25	Mean
1	73.4 e	92.6 c	93.9 bc	86.6 b
7	84.5 d	95.1 b	97.3 a	92.3 a
Mean	79.0 c	93.9 b	95.6 a	89.5

LSD at 0.05 Day = 0.02 Medium volume = 1.33 Day*Medium volume = 1.88

Different letters indicate significant differences ($P \leq 0.05$) based on LSD test.

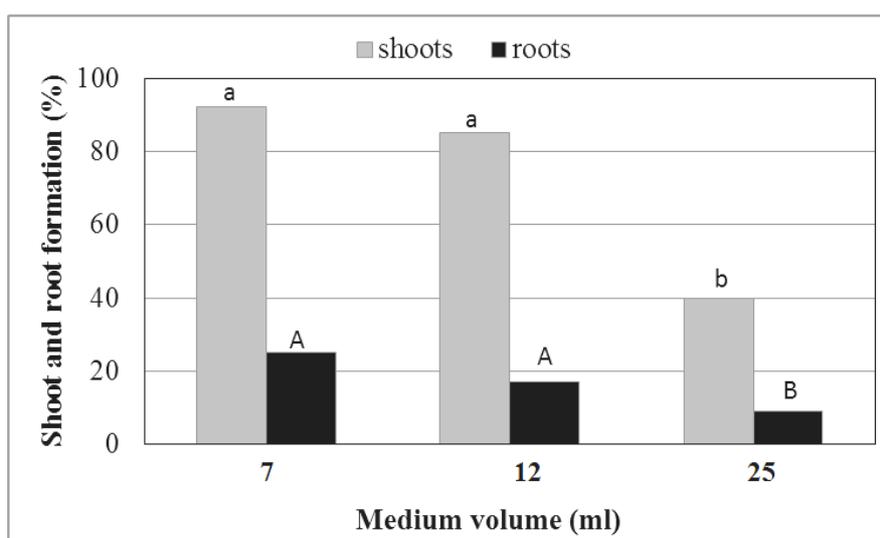


Fig. 5. Effect of volume of water-agar-medium on development of encapsulated nodal segments (MS, 90 g l⁻¹ sucrose, 2 mg l⁻¹ IBA in bead gel matrix) of *R. hybrida* 'Kardinal' after 7 weeks. Different letters indicate significant differences (Chi square test, Pearson, $P < 0.05$).

In another experiment, the bead volume was increased from 85 to 105 μ l to improve the nutrient supply (Fig. 6). Indeed, the increase in root regeneration was improved from 40 to 70% whereas shoot development was not significantly affected and ranged between 30 and 40%. The observed inhibition of shoot develop-

ment could be due to the higher IBA concentration used in this experiment. Therefore, using MS with 1 mg l⁻¹ IBA and 90 g l⁻¹ sucrose as an artificial endosperm resulted in the highest conversion (50 %) of encapsulated nodal segments of *R. hybrida* 'Kardinal' on a water-agar-medium.

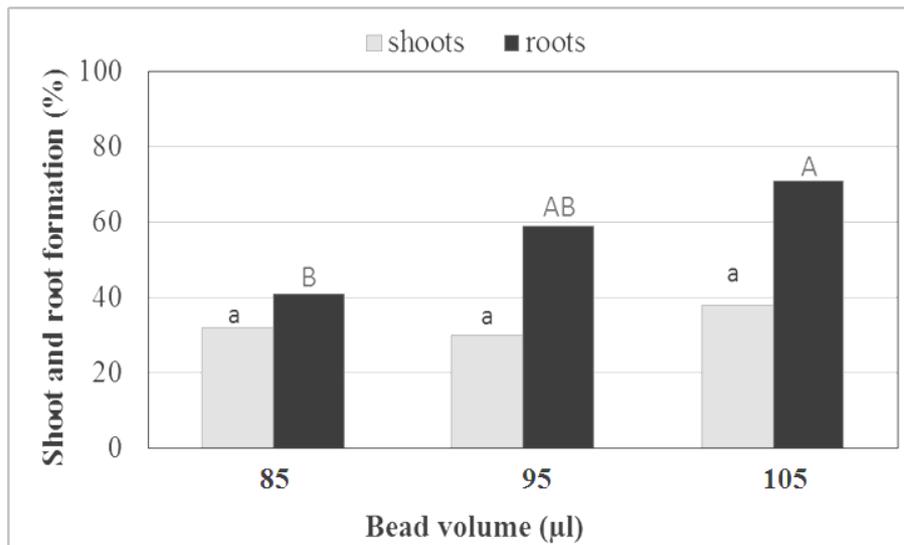


Fig. 6. Effect of bead volume on development of encapsulated nodal segments (MS, 90 g l⁻¹ sucrose, 2 mg l⁻¹ IBA in bead gel matrix) of *R. hybrida* 'Kardinal' after 7 weeks. Different letters indicate significant differences (Chi square test, Pearson, P<0.05).

Effect of sucrose enrichment of the propagation medium

Because in *R. hybrida* 'Kardinal' it was not possible to supply enough nutrients within the beads, the effect of pre-treatment of the shoot cultures by cultivation on media with enhanced sucrose concentration (up to 50 g l⁻¹) has been investigated. Data showed that increasing sucrose concentration in the propagation medium improved root regeneration of rose encapsulated nodal segments with up to 80% whereas the shoot development was reduced from 60 to 10% at the same time (Fig. 7). Tawfik *et al.* (2018) reported that medium

with pH at 5.5 supplemented with 50 g l⁻¹ sucrose produced the highest significant value of rooting (85%) and enhanced root formation in 7.5 days. Similar observations were obtained by Mamiya and Sakamoto (2000), who found that sugar concentration in the post-culture medium affected the growth of shoots and roots in the conversion process. Lower concentrations of sugar promoted the growth of shoots, but higher concentrations of sugar promoted the growth of roots and inhibited the growth of shoots of somatic embryos in *Asparagus officinalis*.

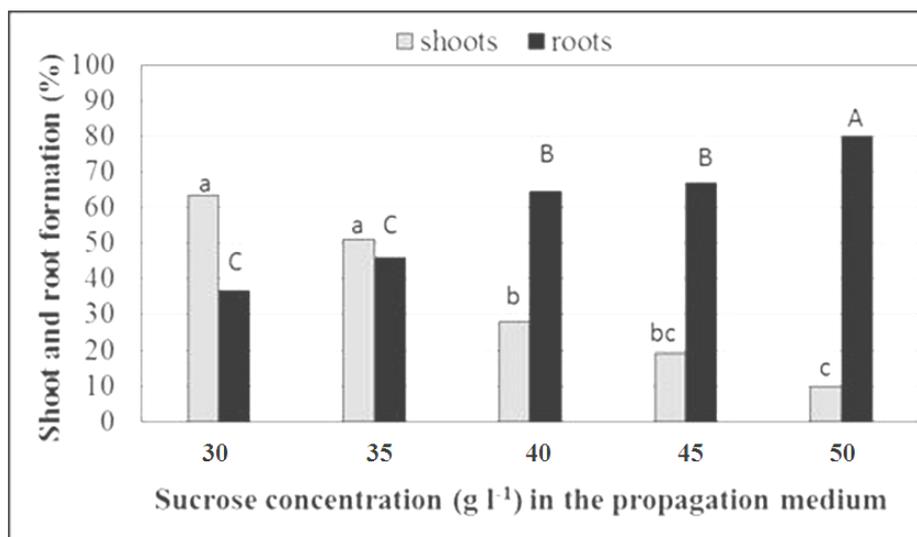


Fig. 7. Effect of sucrose concentration in the pre-treatment propagation medium on shoot and root formation of encapsulated nodal segments (MS, 90 g l⁻¹ sucrose, 2 mg l⁻¹ IBA in bead gel matrix) of *R. hybrida* 'Kardinal' on a water-agar-medium after 7 weeks. Different letters indicate significant differences (Chi square test, Pearson, P<0.05).

Conclusions

The present results clearly indicated that the conversion rate of rose encapsulated nodal segments into plantlets was low on a nutrient-free medium. Maximum conversion rate (ca. 53%) was obtained by use of MS with 1 mg l⁻¹ IBA and 90 g l⁻¹ sucrose as an artificial endosperm on a water-agar medium. The nutrients included in the gel matrix of the calcium alginate bead diffuse rapidly into the nutrient-free substrate (ca. 90% nutrients loss after 24h incubation). The remaining nutrients were not sufficient to stimulate shoot development and root regeneration of cultures with high nutrient demands like rose. In this study, the nutritional status of the beads could be slightly manipulated by reducing the volume of growing media or increasing the bead volume. Therefore, it is suggested that further investigations should be carry out to improve conversion rate of rose en-

capsulated nodal segments into plantlets on a nutrient-free medium.

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تحول العقد الساقية المكبسلة في نبات الورد صنف كاردينال إلى نباتات كاملة معمليا

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المخلص

أجريت هذه الدراسة بجامعة همبولدت ببرلين، قسم نظم نباتات البساتين، ألمانيا أثناء موسمي ٢٠١٢ و ٢٠١٣ بهدف تحديد الاحتياجات الغذائية اللازمة لتحول العقد الساقية المكبسلة في نبات الورد صنف كاردينال إلى نباتات كاملة وأيضا معرفة كمية الفقد في الغذاء من الكبسولات أثناء زراعتها على بيئة خالية من العناصر الغذائية (بيئة الأجار مع الماء). حيث تمت زراعة الأفرع على بيئة موراشيخ وسكوج المعدلة. ولإجراء عملية الكبسلة، تم عزل ٤-٥ ملم من العقد الساقية وذلك بعد ثلاثة أسابيع من إعادة الزراعة. بعدها تم وضع العقد الساقية في محلول الجينات الصوديوم بتركيز ٣% والمخفف في بيئة موراشيخ وسكوج المعدلة مع استخدام تركيزات مختلفة من السكر (٣٠-٩٠ جم/لتر) وتركيزات مختلفة من إندول حمض البيوتيريك (٥, ٢٠, ٥٠, ١٠٠, ١٥٠, ٢٠٠, ٣٠٠, ٤٠٠, ٥٠٠, ٦٠٠, ٧٠٠, ٨٠٠, ٩٠٠, ١٠٠٠ ميكرومول مع وجود العقد الساقية، وذلك في محلول كلوريد الكالسيوم بتركيز ٧٥ ملليمول مذاباً في نفس مكونات بيئة محلول الجينات الصوديوم وذلك لمدة ٣٠ دقيقة من أجل عملية التقسية. أظهرت النتائج أنه بالفعل بعد مرور ٢٤ ساعة من زراعة العقد الساقية المكبسلة على بيئة الأجار مع الماء تم فقد أكثر من ٩٠% من النترات خارج الكبسولات. كما أوضحت النتائج أنه عند خفض حجم بيئة الأجار مع الماء من ٢٥ مل إلى ٧ مل أدى إلى نقص في معدل فقد الغذاء وتحسن ملحوظ في تكوين الفروع من ٤٠ إلى ٩٠%. كما أمكن تحسين معدل خروج الجذور على العقد الساقية المكبسلة من ٤٠ إلى ٨٠% بزيادة حجم الكبسولة من ٨٥ إلى ١٠٥ ميكرومول أو عن طريق زيادة تركيز السكر من ٣٠ إلى ٥٠ جم/لتر في بيئة النكاتر. أعلى معدل تحول للعقد الساقية المكبسلة لنبات الورد على بيئة الأجار مع الماء بلغ حوالي ٥٠% عند إضافة كل من إندول حمض البيوتيريك بتركيز ١ ملجم/لتر والسكر بمعدل ٩٠ جم/لتر إلى مكونات الإندوسبرم الصناعي.