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The effect of medium and plant growth regulators on micropropagation of Dog rose (*Rosa canina* L.)

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Abstract
Dog rose (*Rosa canina* L.) is one of the most important ornamental and medicinal plants which are used as a rootstock for ornamental roses such as *Rosa hybrid* and *Rosa floribunda*. *In vitro* propagation of rose has a very important role in rapid multiplication of species with desirable traits and in production of healthy and disease-free plants. Micropropagation of *Rosa canina* L. was revised, using its nodal segments under different combinations of BAP (0, 0.5, 1, 1.5 and 2 mgl^{-1}), GA_3 (0 and 0.5 mgl^{-1}) and NAA (0 and 0.5 mgl^{-1}) on Murashige and Skoog (MS) and Van der Salm medium (VS) in proliferation stage and using different combinations of NAA and IBA (0, 0.3, 0.6 and 0.9 mgl^{-1}) and ½ VS medium in rooting stage. The highest shoot proliferation was obtained on VS medium containing 2 mgl^{-1} BAP. Furthermore, the highest root induction obtained in ½ VS containing 0.6– 0.9 mgl^{-1} of NAA or IBA. The present study presents an *in vitro* protocol for *R. canina*.
Keywords: Micropropagation, Native plants, Plant growth regulators (PGRs), *Rosa canina* L.

Introduction

Rosa canina L. (belongs to *Rosaceae* family) is a medicinal plant that its fruits have many essential medicinal properties unknown for many people especially in Iran. However, this plant can be produced commercially and its orchards can be established like other fruit trees (12, 18, 19). Fruits of rose species are rich in minerals, vitamins (A, B1, B2, B3, C and K), sugars, phenolic compounds, carotenoids, tocopherol, bioflavonoid, tannins, organic acids, amino acids, volatile oils, vanillin and other photochemicals such as antioxidant and antimicrobials. Medicinal properties and benefits of roses are: nutrient, mild laxative, mild diuretic, mild astringent, carminative, ophthalmic, tonic and verminfuge (18, 19). Dog rose has also been used as a rootstock for ornamental roses (8, 9, 10). More than 200 species are present in the genus *Rosa* (21) from which 14 wild species are found in Iran. Traditionally, most roses are

heterozygous and do not breed true to type. Commercial propagation of roses is usually done by cutting, although they can be propagated by budding and grafting, which are difficult and undesirable processes (4). Conventional rose propagating methods are very slow and time consuming. Tissue culture is becoming increasingly popular as an alternative to conventional rose propagation methods (16). Micropropagation of Rosa species is carried out through the meristem-tip, shoot-tip and axillary buds culture. In recent years, several reports have been published on *in vitro* proliferation of roses, and have shown that tissue culture provides an alternative method for rapid multiplication (13). 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 off and field transfer of tissue culture raised plants (11). The presence of cytokinin in the culture medium helped in the year round multiplication of shoots in hybrid roses (17). Although the rate of growth was found to be very slow. They observed a high percentage of bud break in a hormone-free medium within 10-12 days. However, Media supplemented with BAP or BAP+GA_3 resulted in early bud break within 6-8 days of culture with enhanced rates of shoot multiplication. The addition of BAP (2.0-3.0 mgl^{-1}) as the only growth regulator in the culture medium resulted in feeble callusing at the cut ends of the explants and the shoot elongation was considerably slow in a 60-days culture period and the explants response varied from 63 to 80%. Incorporation of GA_3 at low concentrations (0.1-0.25 mgl^{-1}) in the BAP supplemented medium improved explants response up to 95%. BAP was the most effective growth regulator in stimulating shoot proliferation. Further, the use of 3 types of auxins in combination with BAP showed that NAA was more effective than IAA or IBA in the production of multiple shoots (15, 20). The objective of this study was to investigate the best combinations and media for *in vitro* micropropagation of *Rosa canina* L. as rootstock for grafting.

Materials and methods

Sterilization of explants:

When the buds obtained chilling requirement in February 2012, axillary buds of *R. canina* (grown at the Botanical Garden of Ferdowsi University of Mashhad, Iran) were cut and washed with running tap water (1h), then decontaminated with 70% (w/v) ethanol (30s) and sodium hypochlorite (2.5%) (15min). All explants were washed three times with sterile distilled water.

Shoot proliferation:

In this experiment, shoot explants with 3 axillary buds and 2 cm in length were transferred to 2 different media: MS and VS medium. 20 treatments with 8 replications (one explant in each glass vials) were considered for each medium (MS and VS) in this stage. Different combinations of BAP (0, 0.5, 1, 1.5 and 2 mgl^{-1}), GA_3 (0 and 0.5 mgl^{-1}) and

NAA (0 and 0.1 mgl^{-1}) were considered for MS and VS media. The shoot number, shoot length, shooting percentage, leaf number and percentage of green leaves were recorded after 60 days. The pH of media was adjusted to 5.8 before adding 8 grl^{-1} agar. Media were autoclaved for 15min at 121 C° and 1.2 kgf/cm pressure. All the *in vitro* cultures were placed under 16/8h light/dark cycle in a growth chamber and were maintained at 24±1 C°.

***Rooting stage*:**

New shoots (1-2 cm in height) were transferred VS and ½ VS media containing different hormonal concentrations of IBA and NAA (0, 0.3, 0.6 and 0.9 mgl^{-1}) for rooting. Root length, root number and the percentage of root induction were recorded after 30 days.

Acclimatization of plantlets:

The plantlets were cultivated for 5 weeks in plastic glasses containing sterile mixture of Peat/Perlite (3/1). The plantlets were put in growth chamber under a 16/8-hours photoperiod, at a temperature of 23/25 C^0 (night/day) and 80% relative humidity. The shoot survival percentage was recorded after 30 days.

Experimental design and statistical analysis

The shoot proliferation experiment was performed in completely randomized design (CRD) with 40 treatments (composition of BAP (0, 0.5, 1, 1.5 and 2 mgl^{-1}), GA_3 (0, 0.5 mgl^{-1}) and NAA (0 and 0.1 mgl^{-1}) in MS and VS medium) and 8 replications (one explant in each replication). Rooting experiment was carried out in a completely randomized factorial base on design with 16 treatments (composition of IBA and NAA (0, 0.3, 0.6 and 0.9 mgl^{-1}) in VS and ½ VS medium) and 15 replications (one explant in each replication). Data were analyzed with SPSS software (version 19) and the comparison of means was performed by Duncan's new multiple range test at $P \leq 0.05$. Before carrying out ANOVA the percentage data were transformed using angular transformation (Arc Sin$\sqrt{}$%).

Results:

Shoot proliferation:

The results showed that the effect of treatments (type and concentrations of PGRs) on vegetative traits of *R. canina* in proliferation stage was significant ($P \leq 0.05$) (Table 1). The maximum number of new leaves (19.96), shoot per explant (4.21) and shoot percentage (41.10%) were produced on the medium containing 2 mgl^{-1} BAP (Figure 1A, 1B, 1C), whereas the maximum percentage of green leaves (84.84%) and the highest increase in plantlet height (1.27 cm) were obtained on the medium containing 1.5 mgl^{-1} BAP (Figure 1D, 1E). Also, the effect of medium on vegetative traits in proliferation stage was significant ($P \leq 0.05$) (Table 1). The lowest shoot multiplication was observed on MS medium while the highest shoots were formed on VS medium (Table 2).

Table 1. Analysis of Variance (mean of square) of *R.canina* in proliferation stage (P≤0.05).

Source of Variation	Degree of Freedom (df)	Shoot number	Leaf number	Shoot length	Percentage of green leaves	Shoot percentage
Medium	1	4.82 ^{ns}	319.8 **	4.05 **	8318.1 **	308.40 ^{ns}
Hormone	19	9.56 **	267.84 **	0.723 **	4395.11 **	512.98 **
Error	280	1.7	35.86	0.197	407.62	97.32

ns: non- significant

*,**: significant at P< 0.05 and 0.01, respectively.

Kim *et al*. (6) indicated that *in vitro* shoot proliferation and multiplication of roses are largely based on medium formulations containing cytokinins as a major plant growth regulators, although low concentrations of auxins or GA_3 are also essential. The results of the present study demonstrated that inclusion of 0.5 $mgl^{-1}GA_3$ to the culture media didn't increase the number of axillary shoots and stem height in all of the BAP concentrations. The results showed that in some of the traits the differences between the treatments were not significant, however, the maximum number of axillary shoots were significantly higher in the VS medium containing 2 mgl^{-1} BAP. As the concentration of BAP was lower than 2 mgl^{-1}, a reduced growth rate was noted. 2 types of MS and VS medium were also used in this experiment and the results showed that the best plantlets (in proliferation stage) obtained in VS medium because of the alleviating effect of iron deficiency in leaves.

Table 2. Effect of different media on some vegetative traits of *R.canina* in proliferation stage.

medium	shoot number	leaf number	shoot length (cm)	green leaves (%)	Shooting (%)
MS	2.15 a	8.37 b	0.768 b	46.29 b	26.25 a
VS	2.4 a	10.37 a	0.993 a	56.49 a	28.21 a

Different letters indicate significant difference according to Duncan Test (*P*<0.05)

Root initiation and acclimatization

ANOVA of the effect of two media on root number, root length and rooting percentage revealed significant differences (P≤0.05) (Table 3). The results showed that the effect of concentrations and the types of PGRs on root number and rooting percentage were significant but there was no difference in root length (P≤0.05) (Table 3). The highest root length (1.85 cm), root number (2.28) and rooting percentage (24.75%) were recorded in ½ Vs medium (Table 4). The maximum root number (2.14) and rooting percentage (23.08%) were also observed in 0.6 and 0.9 mgl^{-1} IBA or NAA, respectively (Table 5). The results showed that there are differences between IBA and NAA treatments in root length in comparison with control, while significant differences in root number and rooting percentage was recorded. Therefore, the PGRs concentrations had more importance than the PGRs types in rooting stage.

The regenerated shoots cultured on VS medium containing ½ and full strength of VS (macro, micro elements and vitamins) showed different responses to rooting after 4 weeks of culture. The best rooting were obtained on medium containing ½ strength of VS macro, micro salts and vitamins. Acclimatization of the rooted plantlets were easily carried out at ±24 C° and the relative humidity of 80% during initial stages of development and after that it was gradually reduced to 40%. After 4 weeks of culture acclimatized plants were transferred to greenhouse for flowering.

Table 3. Analysis of variance (mean of square) on some traits of *R. canina* in rooting stage ($P \leq 0.05$).

Source of variation	Degree of freedom	Root number	Root length	Rooting percentage (%)
PGRs type (A)	1	13.43 *	0.74 ns	882.25 *
PGRs concentration (B)	3	14.46 *	0.48 ns	871.28 *
Culture Medium (C)	1	85.12 **	45.88 **	6036.00 **
A x B	3	11.69 *	0.85 ns	704.061 *
A x C	1	36.85 **	18.07 **	2523.17 **
B x C	3	19.03 **	9.15 *	1312.36 **
A x B x C	3	5.23 ns	3.33 ns	324.209 ns
Error	224	4.54	3.44	295.96

ns: non- significant

*,**: significant at P< 0.05 and 0.01, respectively.

Table 4. Effect of different media on some traits of *R.canina* in rooting stage.

Medium Culture	Root number	Root length (cm)	Rooting percentage (%)
Vs	1.01 b	0.922 b	14.03 b
½ Vs	2.28 a	1.85 a	24.75 a

Different letters indicate significant difference according to Duncan Test (P<0.05)

Discussion:

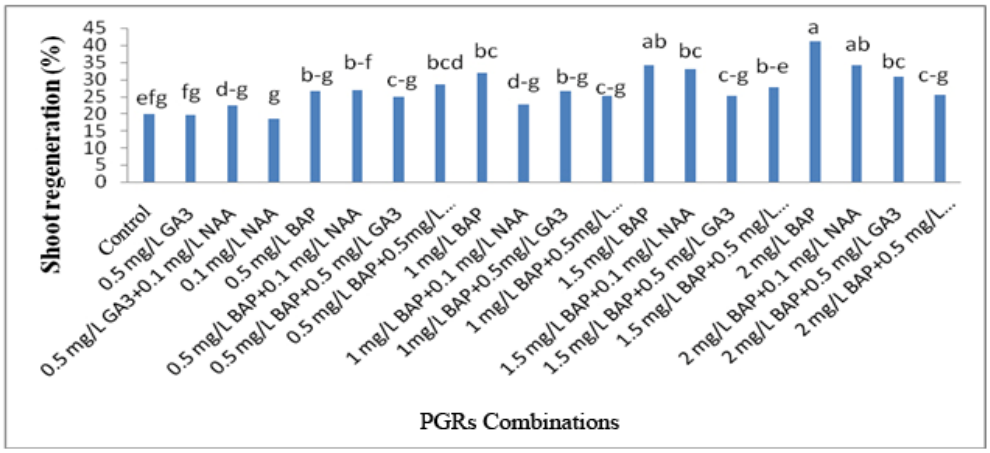
Tissue culture techniques are used extensively for growing plants commercially. In this study, according to the responses of explants cultured on two media, VS and ½ VS were relatively the best and the most appropriate treatments for shoot and root formation. For initiation of aseptic cultures, a thorough knowledge of the physiological status and the susceptibility of the plant species to different pathological contaminants are required. So, utilization of 70% (w/v) ethanol (for 30s), and sodium hypochlorite (2.5%) (for 15min) will be effective. Other researchers have reported a high percentage of breaking bud dormancy on hormone-free medium within 10-12 days, but the growth rate was very low in roses (17). In our study the effects of different concentrations of BAP (0 to 2 mgl^{-1}), ,GA_3 (0 and 0.5 mgl^{-1}) and NAA (0 and

0.1 mgl^{-1}) were obvious on bud break and growth in VS medium, however, the growth rate was lower on MS and VS medium without growth regulators. The VS medium with additive Fe (FeEDDHA chelat) was also better than MS medium (with EDTA chelat) in all stages of micropropagation of this plant.

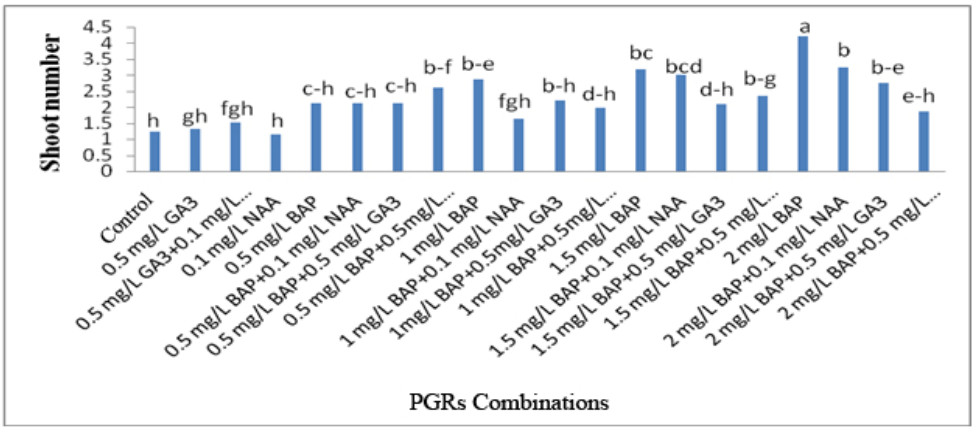
Table 5. Effect of concentrations of PGRs on rooting traits of *R.canina*.

Concentration (mg/L)	Root number	Root length (cm)	Rooting percentage
0	0.77 b	1.42 a	12.29 b
0.3	1.4 ab	1.27 a	17.78 ab
0.6	2.14 a	1.38 a	23.08 a
0.9	1.83 a	1.48 a	20.86 a

Different letters show significant differenced according Duncan Test ($p<0.05$).



(A)



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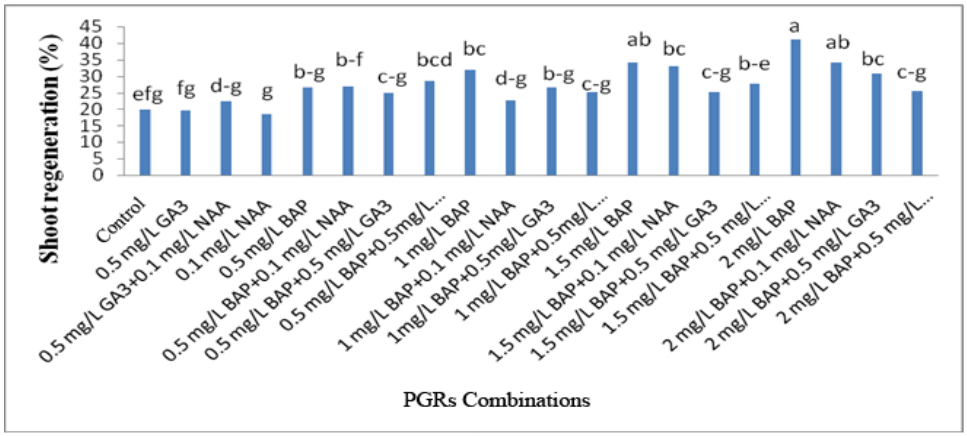

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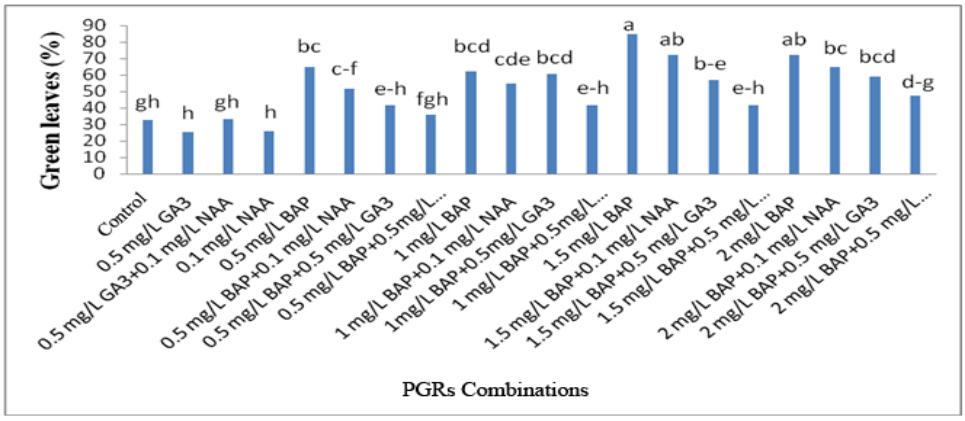

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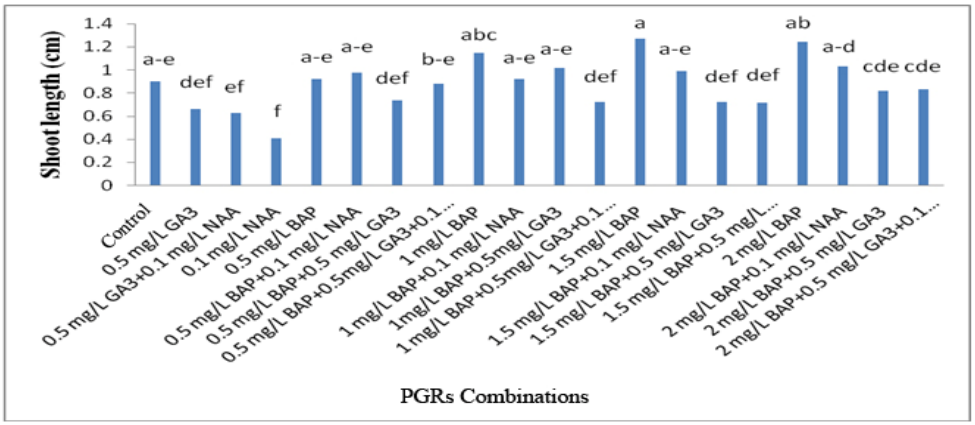

(E)

Figure 1. Effect of Hormones (type and concentrations of PGRs) on vegetative traits of *R.canina* in proliferation stage, showing; A) Average number of new leaves B) Average number of shoot per explant C) Average Shoot percentage (%) D) Average percentage of green leaves (%)E) Average Shoot length (cm).

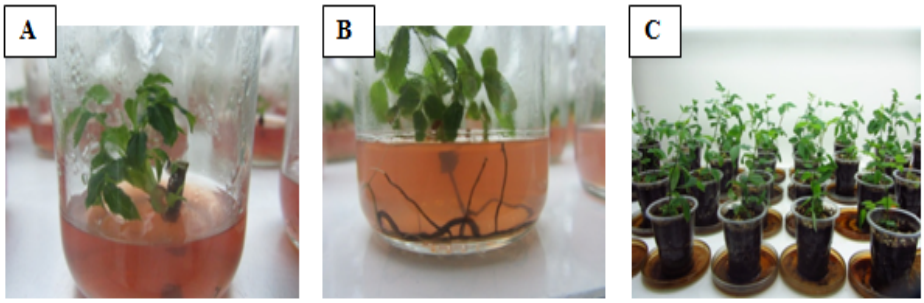

Figure 2. Different stages of micropropagation of *Rosa canina* L: A) Proliferation stage B) Rooting stage C) Acclimatization stage.

In vitro shoot multiplication relies largely on medium formulations containing BAP as the major PGRs in combination with a low concentration of NAA (22). In the present study, 2 mgl^{-1}BAP was the optimum treatment for *in vitro* multiplication of *Rosa canina* L.. The result is different from the findings on optimal BAP concentration (4.4– 13.2 µM) (2). BAP is needed for proliferation of *Rosa canina* L. plants, 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 and this is not in line with our results. When the culture medium contains NAA at higher concentration, the bud can form more callus from the base section, which will greatly affect the young seedlings for absorption of water and nutrition, and thus inhibits their growth. The inhibition is especially obvious when NAA concentration is increased to 0.5 mgl^{-1}. Auxin is a rooting hormone and the application of synthetic auxin i.e. IBA might have increased the biosynthesis of indole acetic acid (IAA) or could act as synergistic to IAA. (3). Another possible reason for higher rooting and early root initiation by IBA might be involvement of IBA in ethylene biosynthesis (1). It has been suggested that auxin induced ethylene may induce adventitious root formation instead of action of auxin itself (14). A high concentration of GA_3 (0.5 mgl^{-1}) in present study caused the chrolosis and the death of young shoots, which appeared waterlogged and that is in line with Xing *et al* (22). The best results in rooting stage achieved with 0.6 or 0.9 mgl^{-1}IBA/NAA in ½ VS medium. Decreasing medium salt strength of MS medium generally increases rooting in rose micropropagation. (5) The same results were reported in *R.hybrida* cv. Peace (7) and *R.rugosa* (22). Our study yielded a practical protocol for efficient axillary bud multiplication from *Rosa canina* L. explants. Here we demonstrate high-efficiency micro-propagation of *R. canina*. In conclusion, the present study provides an *in vitro* propagation protocol for *R. canina*. At the proliferation stage, maximum number of axillary shoots was achieved in the medium containing 2 mgl^{-1}BAP. At the rooting stage, 0.6 and 0.9 mgl^{-1} IBA/NAA were effective.

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تأثیر محیط کشت و تنظیم کننده‌های رشد بر ریزازدیادی نسترن وحشی (*Rosa canina* L.)

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چکیده:

نسترن وحشی (*Rosa canina* L.) یکی از گیاهی زینتی و دارویی با اهمیت است که به عنوان پایه برای اکثر رزهای زینتی نظیر رزهای هیبرید و رزهای خوشه‌ای مورد استفاده قرار می‌گیرد. تکثیر درون شیشه‌ای رز نقش بسیار مهمی در تکثیر سریع گونه‌هایی با خصوصیات مطلوب و تولید گیاهان سالم و عاری از بیماری دارد. در این تحقیق ریزازدیادی گونه نسترن وحشی با استفاده از ریزنمونه گره در غلظت‌های مختلف تنظیم‌کننده‌های رشد در مرحله پرآوری شامل ترکیبات بنزیل آمینو پورین (۰، ۰/۵، ۱، ۱/۵ و ۲ میلی‌گرم در لیتر)، جیبرلیک اسید (۰ و ۰/۵ میلی‌گرم در لیتر) و نفتالین استیک اسید (۰ و ۰/۵ میلی‌گرم در لیتر) در دو محیط کشت موراشی و اسکوگ (MS) و وندر سالم (VS) و در مرحله ریشه‌زایی شامل دو تنظیم کننده نفتالین استیک اسید و ایندول بوتیریک اسید (۰، ۰/۳، ۰/۶ و ۰/۹ میلی‌گرم در لیتر) در دو محیط کشت VS و VS ½ در شرایط درون شیشه‌ای مورد بررسی قرار گرفت. نتایج نشان داد که بیشترین پرآوری شاخه در محیط کشت VS و در غلظت ۲ میلی‌گرم در لیتر BAP بدست آمد. علاوه بر این، بیشترین القای ریشه‌زایی در محیط VS ½ و در غلظت ۰/۹ تا ۰/۶ میلی‌گرم در لیتر IBA یا NAA بدست آمد. تحقیق حاضر پروتوکل تکثیری بهینه برای گونه نسترن وحشی را ارائه می‌دهد.

کلمات کلیدی: ریزازدیادی، گیاهان بومی، تنظیم کننده‌های رشد، نسترن وحشی (*Rosa canina* L.).