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Full Length Research Paper

Effects of inorganic nitrogen source and NH_4^+: NO_3^- ratio on proliferation of dog rose *(Rosa canina)*

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Dog rose (*Rosa canina*) is one of the most important ornamental and medicinal plants which are used as rootstock for ornamental roses as *Rosa hybrida* and *Rosa floribunda*. The effects of different concentrations of NH_4NO_3 (10-15 and 20 mM), KNO_3 (15-20 and 25 mM) and NH_4:NO_3 ratios (10:25, 10:30, 10:35, 15:30, 15:35, 15:40, 20:35 and 20:45) was investigated on proliferation stage of dog rose cultivated on Ms media with total nitrogen levels (35; 40, 45, 50, 55, 60 and 65 mM). The relative amounts of NH_4^+ and NO_3^- ions influenced the tested parameters. The highest shoot length and most node numbers were obtained with 20:45 NH_4^+:NO_3^- ratios. Chlorosis leaf number was less with 15:35 and 20:45 NH_4^+:NO_3^- ratios. When NO_3^- was higher than NH_4^+, shoot length and node numbers were produced more and chlorosis leaf number was decreased. This study also demonstrates that with increasing nitrogen levels, the growth of shoots and node numbers were more but leaf chlorosis was decreased, therefore proliferation was better. The percentage of axillary shoot was affected by NH_4NO_3 concentrations and it was highest in 20 mM concentration, but with increasing KNO_3, the leaf necrotic and feeble callusing percentage at the cut ends of explants were more and lower in row.

Key words: Dog rose, *in vitro* culture, MS nitrogen source, NH_4^+: NO_3^- ratio, proliferation.

INTRODUCTION

Rosa canina is a medicinal plant that its fruits have many important medicinal properties but unknown for many people's especially in Iran. However, this crop can be produced commercially and its orchards can be established such as other fruit trees (Sharafi, 2010; Iuncna, 2005). Fruits of the rose species are rich in minerals, vitamins (A, B1, B2, B3, C and K), sugars, phenolic compounds, carotenoids, tokepherol, bioflavonoid, tannins, organic acids, amino acids, volatile oils, vanillin and other photochemical such as antioxidant and antimicrobials. Medicinal properties and benefits of Rose are: Nutrient, mild laxative, mild diuretic, mild astringent, carminative, ophthalmic, tonic and verminfuge (Sharafi, 2010). Active ingredients of *R. canina* can protect cold, flu, infectious diseases, vitamin C deficiency and fever (Iuncna, 2005). Commercial propagation of roses is usually done by cutting, although they can also be propagated by budding and grafting, which is difficult, undesirable and tedious process (Horn, 1992). Micropropagation of *Rosa* species is done through the meristem tip culture, shoot tip culture and axillary buds (Khosh-Khui and Sink, 1982; Skirvin et al., 1990; Rout et al., 1999). 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 (Horn et al., 1988). This technique is a tool for propagation of many plant species and is important for mass propagation (George, 1993). Although most rose micropropagation studies have used a solid agar-gelled medium; Chu et al. (1993) and Nikbakht et al. (2005) have reported that shoot proliferation of roses is higher on liquid medium rather than solidified medium because; nutrient absorption may be facilitated in this kind of medium (George, 1993). Most plant tissue culture media, which are characterized by high levels of nitrogen in the forms of ammonium nitrate and potassium nitrate, are still based on the nutrient component of Murashige

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Table 1. Types of diferent NH_4^+:NO_3^- ratios and total nitrogen.

	Treatments								
NH_4^+:NO_3^- ratio (mM)	10:25	10:30	10:35	15:30	15:35	15:40	20:35	20:40	20:45
Total nitrogen (mM)	35	40	45	45	50	55	55	60*	65

*Total nitrogen and NH_4^+:NO_3^- ratio are according to standard MS medium.

Table 2. Analysis of variance table.

Sources	Df	Mean square					
		Shoot length (cm)	Node number	Axillary shoot (%)	Chlorosis leaf number	Necrotic leaf number	Feeble callusing (%)
NH4NO3	2	100^{**}	57.75^{**}	0.88^{*}	1.63^{ns}	1.3^{ns}	18^{ns}
KNO3	2	15.6^{ns}	12.20^{ns}	0.14^{ns}	4.13^{*}	9.2^{**}	72^{**}
KNO3×NH4NO3 (NH_4^+:NO_3^-ratio)	4	53^{**}	23.52^{*}	0.37^{ns}	3.51^{*}	0.95^{ns}	45^{ns}
Error	72	1.60	7.02	0.20	1.30	0.21	0.11

Levels of significance are: * $p<0.05$, ** $p<0.01$ and Ns: non significant by ANOVA.

and Skoog (MS) (1962). The form and amount of nitrogen in the *in vitro* medium have significant effects on the rate of cell growth, differentiation and totipotency (Kirby et al., 1987). In the media which nitrate is the only source of nitrogen, often more alkaline state is established with time. This situation slightly improved by adding ammonium salt. pH controlling not only is reason for using both form of nitrate and ammonium in the medium, but also free ammonium ions are toxic. Also optimum ratio of NH_4^+:NO_3^- is effective on morphogenesis of many plants (Bonga and Von Aderkas, 1992). Sharafi (2010) was found the best *in vitro* medium for hawthorn and dog rose pollen germination and tube growth in different researches. In this research the effect of inorganic nitrogen sources (NH_4NO_3 and KNO_3) were studied on proliferation stage of dog rose.

MATERIALS AND METHODS

After disappearing chilling requirement of buds in February, 2010, axillary buds of dog rose (which are grown in botanical garden of University of Tabriz) were cut and then placed under running tap water (for 1 h) and washed for 15 min, with Tween 80 (0.1%) and then decontaminated with 70% Ethanol (for 5 min), 0.1% (w/v) solution of Mercury (II) Chloride (for 2 min), Sodium Hypochlorite (for 20 min). Finally; all the explants were washed three times by sterile distilled water. Cefotaxime 250 mg. l^{-1} and Tetracycline 100 mg. l^{-1} were used through direct addition on establishment medium for bacterial decontaminations. However; explants were transferred to the prepared medium after sterilization. MS (Murashige and Skoog's, 1962) basal medium was used for explants establishment stage which was contained 30 g. l^{-1} sucrose, 0.8% Agar, 1 mg. l^{-1} GA_3 and 1 mg. l^{-1} BAP. In proliferation medium, MS medium was modified with combination of both concentrations of NH_4NO_3 (10, 15 and 20 mM) and KNO_3 (15, 20 and 25 mM) at the ratio of NH_4^+:NO_3^- (Table 1) where supplemented with 20 g. l^{-1} sucrose, 10 g. l^{-1} sorbitol, 0.8% Agar, 5 mg. l^{-1} $AgNO_3$, 1 mg. l^{-1} GA_3 and 3 mg. l^{-1} BAP. The pH of media was adjusted to 5.8 then culture medium was autoclaved at 121°C at 105 kPa for 20 min. Explants were incubated in the culture room at temperature of 25 ± 2°C and photoperiod of 16 h light and 8 h dark cycle. In proliferation stage, after four weeks shoot length, node number, axillary shoot percentage, chlorosis leaf number, necrotic leaf number and feeble callusing percentage at the cut ends of explants were recorded. All experiments were carried out as in completely randomized design (CRD) with 8 replications. Data were analyzed with SPSS software (ver. 16) and comparison of means was performed by Duncan's New Multiple Range Test at $P < 0.05$.

RESULTS AND DISCUSSION

Analysis of variance showed that, shoot length significantly influenced by different concentrations of NH_4NO_3 and NH_4NO_3× KNO_3 interaction (NH_4^+:NO_3^- ratio) ($p<0.05$) (Table 2). The highest shoot length was observed in 20:45 ratio of NH_4^+:NO_3^-. In this ratio the amount of total nitrogen in culture medium was high (65 mM). The lowest shoot length was observed in 15:30 NH_4^+:NO_3^- ratio (total N 45 mM) (Figure 1A). In comparison of 20:45 and 15:30 NH_4^+:NO_3^- ratio; more concentration nitrate ion and high total nitrogen may be the main reason of increasing shoot length. The high levels of ammonium ion may be inhibited growth of shoot length due to decrease the activity of Nitrate Reductase and Glutamate Syntheses Enzymes in producing of amino acids (Gamborg and Shyluk, 1970). So nitrate is the most important form of nitrogen that used by the dog rose. Moreover; results showed that the shoot length was increased with rising of total nitrogen in different treatments. Nitrogen is a major element for amino acid, protein and nucleic acid biosynthesis and has important roles in many metabolism pathways, cell growth and

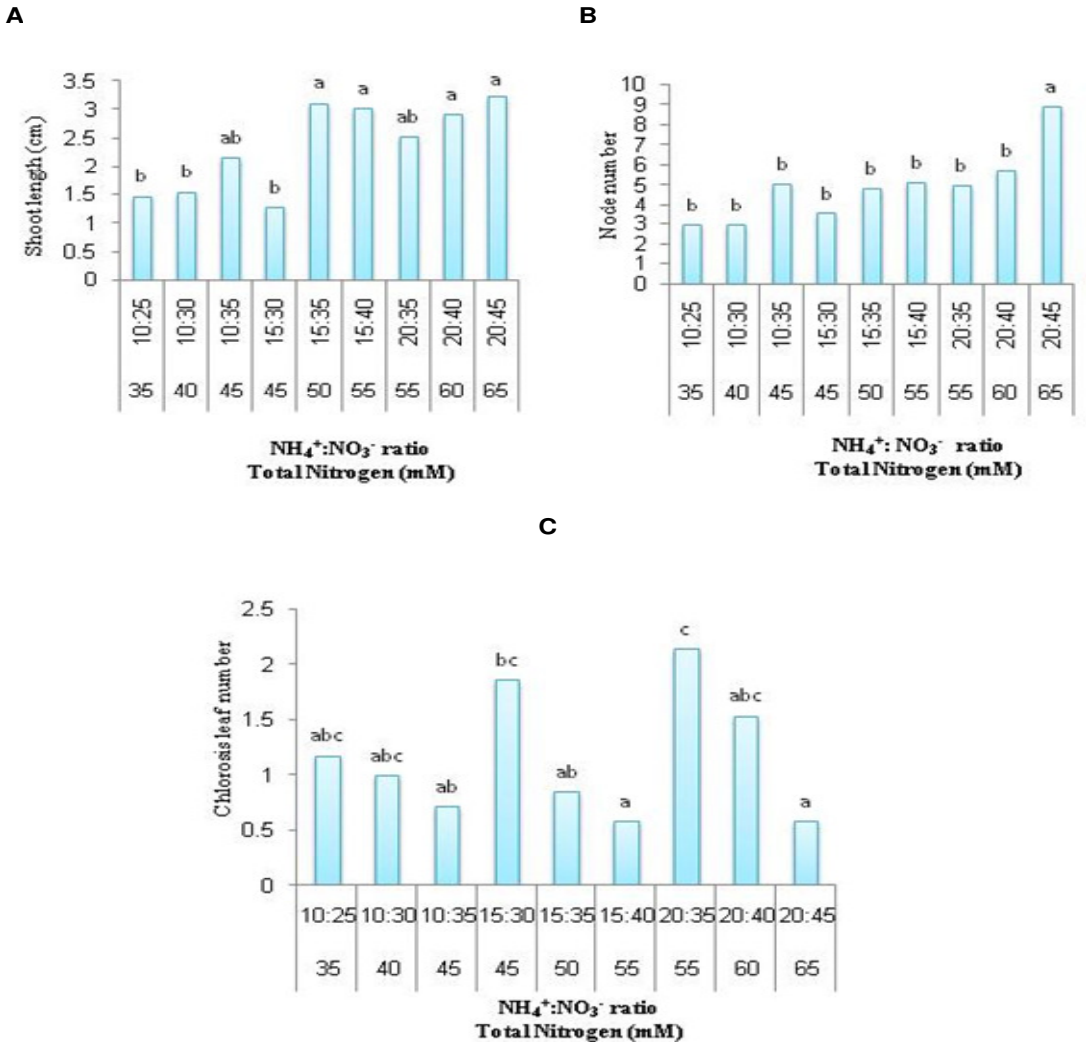

Figure 1. Effect of NH_4^+:NO_3^- ratio (mM) in MS media on the shoot length (A), node number (B), chlorosis leaf number (C) of Dog rose after 4 weeks in culture. Bars with common letters are not significantly different at $p<0.05$, according to LSD test.

differentiation. The deficiency of nitrogen quickly inhibits plant growth and development (Taiz and Zeiger, 2002).

The different concentrations of NH_4NO_3 and interaction of NH_4NO_3× KNO_3 ($p<0.05$) (Table 2) were influenced significantly node numbers. Node numbers in 20:45 NH_4^+:NO_3^- ratio was maximum and it was minimum in 10:30 and 10:25 NH_4^+:NO_3^- ratios (Figure 1B).

Nitrate is essential for axillary meristem and leaves formation. So; omission of nitrate from the medium, inhibit axillary meristem and leaves formation (Ramage and Williams, 2001). Moreover, in 10:25 and 10:30 NH_4^+:NO_3^- ratios less node number was observed, because total nitrogen (35 and 40 mM respectively) was low in comparison with 20:45 NH_4^+:NO_3^- ratio (total N 65 mM). Balancing of NH_4^+ and NO_3^- ions in medium is necessary for better absorption of NO_3^- ion and inhibiting of NH_3 toxicity inside the tissues. Therefore; cell growth is depending on optimum ratio of NH_4^+:NO_3^- in medium (Lenee and Chupeau, 1989). This is in accordance with the results obtained by Ivanova and Van staden in *Aloe polyphylla* (2009).

Axillary shoot percentage was influenced significantly by different concentrations of NH_4NO_3 ($p<0.05$) (Table 2) and interaction of NH_4NO_3 × KNO_3 was insignificantly influenced on axillary shoot percentage. Axillary shoot percentage increased with increasing of NH_4NO_3 from 10 to 20 mM. Minimum and maximum axillary shoot percentage was observed in 10 and 20 mM concentration respectively. There was insignificant difference between two concentrations of NH_4NO_3, 10 and 15 mM, for axillary shoot percentage (Figure 2). Cholorosis leaf number was influenced significantly by different concentrations of KNO_3 and interaction of NH_4NO_3× KNO_3 ($p<0.05$) (Table 2). Highest chlorosis leaf number was observed with

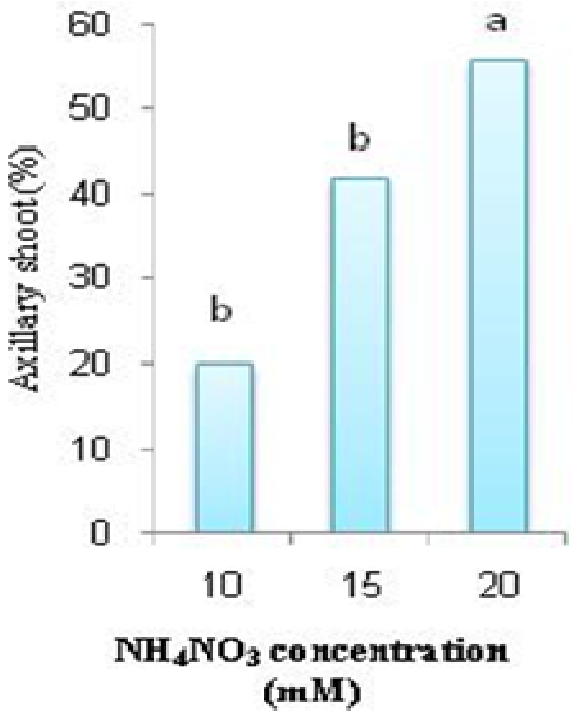

Figure 2. Effect of NH_4NO_3 concentrations in the culture media on the axillary shoot percentage. * Bars with common letters are not significantly different at $p<0.05$, according to LSD test.

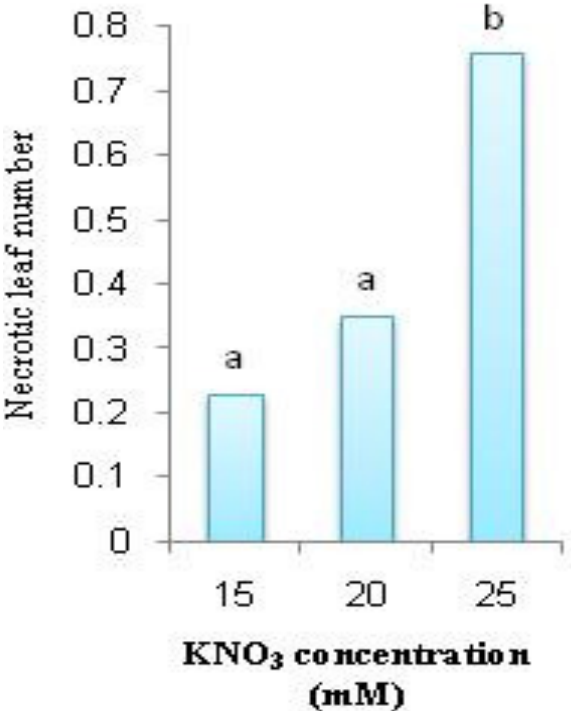

Figure 3. Effect of KNO_3 concentrations in the culture media on the necrotic leaf number. * Bars with common letters are not significantly different at $p<0.05$, according to LSD test.

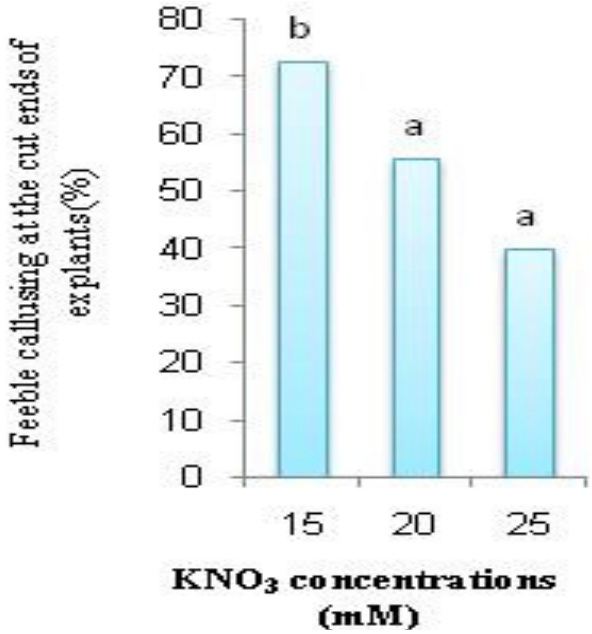

Figure 4. Effect of NH_4NO_3 concentrations in the culture media on feeble callusing percentage at the cut ends of explants. * Bars with common letters are not significantly different at $p<0.05$, according to LSD test.

increase NH_4^+:NO_3^- ratio (Figure 1C). High ratio of NH_4^+:NO_3^- in medium can be reason for leaves chlorosis, so that with cell inside ammonium ion ethylene produced increased and following this phenomenon; senescence of leaves was occurred (Barker et al, 1987). The amount of total nitrogen was reported less important than NH_4^+:NO_3 ratio for cholorosis leaf which may be due to the NH_4^+ and NO_3^- ions role in changing of media pH and loss absorption of nutrient elements (Ramage and Williams, 2001). When total nitrogen on medium is high, some of the leaves are suffering from chlorosis due to total nitrogen deficiency in the medium. Necrotic leaf number was influenced significantly by different concentrations of KNO_3 ($p<0.05$) (Table 2). Interaction of NH_4NO_3× KNO_3 was none significant on necrotic leaf number. Maximum necrotic leaf number was observed in the 25 mM concentration of KNO_3 (Figure 3). High concentration of K^+ in the medium can be lead to leaf necrotic, so that deficiency of calcium occurred with the more K^+ inside tissues and deficiency of calcium can be lead to leaf necrotic (Barker et al, 1987). Different concentrations of KNO_3 were significantly influenced on feeble callusing percentage at the cut ends of explants ($p<0.05$) (Table 2) and interaction of NH_4NO_3× KNO_3 was none significantly influenced on feeble callusing percentage. Minimum bottom callus formation observed at 25 mM concentration of KNO_3 (Figure 4), KNO_3 in high concentration may be inhibited in the form of undifferentiated cells. This result is in accordance with the results obtained by Salehi shanjani (2003) in *Juniperus excels*.

Conclusion

In Dog rose optimum NH_4^+:NO_3^- ratio affected shoot length, the node number and cholorosis of leaf. Ammonium ion was effective on produce axillary shoot and nitrate ion was effective on necrotic leaf number and feeble callusing percentage at the cut ends of explants. Various forms of N supplied overlooked importance of this nutrient in plant growth and morphogenesis *in vitro*.

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