

AXILLARY SHOOT PROLIFERATION AND REGENERATION OF RED ROSE [*ROSA PISIFORMIS* (CHRIST.) D. SOSN. - AN UNDER THREAT EXTINCTION SPECIES ENDEMIC TO TURKEY

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Abstract

In vitro propagation of red rose [*Rosa pisiformis* (Christ.) D. Sosn.] has great importance in rapid proliferation of species with medicinal features and in culture of healthy plants free from diseases. After a successfully pre-sterilization procedure, experiments were maintained by two-phase: multiplication phase and rooting of microshoots phase. Micropropagation of Rose was improved, using its nodal segments under different combination of BAP (0, 1.0, 2.0, 0.6 mg/l), NAA (0, 0.1, 0.2, 0.01 mg/l) on Driver and Kuniyuki (DKW) and MS medium in multiplication phase and using distinct compositions of IBA (0, 1.0, 3.0, 5.0 mg/l) and DKW medium in rooting phase. The maximum number of shoots/per plant (22.6) with the highest number of leaves (222.6) were obtained in DKW medium supplemented with 0.6 mg/l⁻¹ BAP and 0.01 mg/l NAA. The leaf explants were also used to occur callus in MS with different combinations of BAP and NAA. Good callus formation was obtained with 1:2 ratio of BAP:NAA combination in MS medium. Furthermore, the highest root induction (100%) was achieved in DKW medium consisting of 1 mg/l IBA. The rooted plantlets were transferred to the field after acclimatized in greenfield conditions. The present investigation presents an *in vitro* protocol for *R. pisiformis*.

Introduction

Rose hips are wild plants mostly originating in a very wide area in Asia, less often in Europe, North America, and Northwest Africa, Iraq, Northern and Western Iran, Northern Afghanistan and Pakistan including Kashmir and the Commonwealth of Independent States (Ercisli and Esitken 2004, Aghamirov and Farzaliev 2005, Ekinialp *et al.* 2007, Özçelik 2018, Özçelik and Koca 2021) that have medicinal compounds traditionally used in the treatment of various diseases (Wang *et al.* 2022). Turkey is the germination and seed conservation place of many rose species (Ercisli 2005). The exact number of *Rosa* species in Turkey is not known but Nilsson (1972) reported as 24 and Güner (2012) reported as 31. These are distributed over the east, northwest and northeast regions of Turkey, in Gumushane, Tokat, Sivas, Erzincan, Erzurum, Kars and Van provinces. *Rosa pisiformis* (Christ.) D. Sosn. and *Rosa dumalis* BECHST subsp. boissieri (CREPIN) Ö. NILSSON var. *antalyensis* (MANDEN.) Ö. NILSSON are the only **endemic** ones. As being a near threatened plant includes in IUCN Red Data Book categories (Ekim *et al.* 2000), endemic *Rosa pisiformis* (Christ.) D. Sosn is a perennial deciduous which has ornamental value with deep rose petal colour (Ansin 1996, Ercisli 1996) and a medicinal shrub used in diabetes (Dalar 2018, Akkoyun *et al.* 2021) belongs to Rosaceae family widely distributed North East and East Anatolia an Irano-Turanian element. *R. pisiformis* has distributed only at high altitudes (1600-2000) between *Rosa* species so it can not extend in lower altitudes. Because of its reduced distribution area and depressed reproduction, it is graded vulnerable (Ansin 1996).

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Between the rose hip species *R. pisiformis* differs with the highest α -tocopherol content (17.60 $\mu\text{g/g}$) (Yoruk *et al.* 2008), and with high levels of minerals and vitamins. Fruits were used to treat cardiac diseases with antioxidant and antiradical activities (Bayramoğlu *et al.* 2016). *R. pisiformis* fruits have shown also antidiabetic effects *in vivo* on some metabolic enzymes (G6PD, 6PGD, GR, TrxR and GST) in Streptozotocin (STZ) applied diabetic rats (Akkoyun *et al.* 2021). The high content of total phenolics of *R. pisiformis* fruits was the reason of these antioxidant, antimutagenic, anticarcinogenic effects (Ercisli 2007).

Rosa L. taxa are dominantly used for horticulture, food, medicinal, ornamental and aesthetic purposes. Rose is known to be propagated by vegetative methods (Horn 1992), but it is not a convenient way to produce healthy and virus-free rose plants. *In vitro* culturing of roses by various techniques are successfully used as an alternative method of propagation. Among them by shoot regeneration and multiplication technique *R. hybrida* cultivars were proliferated in MS with BAP, NAA or Kinetin (Tawfik *et al.* 2018) and GA_3 (Valles and Boxus 1987, Dohare *et al.* 1991, Singh and Smayal 2000, Carelli and Echeverrigaray 2002, Kim 2003). By the way *R. damascene* species also proliferated with different combinations of BA and IBA (Jabbarzadeh and Khosh-Khui 2005).

Somatic embryogenesis has been reported in *Rosa* sp. since 1967 (Hill 1967). Somatic embryos (SE) can be succeeded from leaves (de Wit *et al.* 1990, Kintzios *et al.* 1999, Kim *et al.* 2004, Estabrooks *et al.* 2007, Vergne *et al.* 2010, Chen *et al.* 2014, Chen *et al.* 2016, Randoux *et al.* 2014, Pehlivan *et al.* 2017). In *Rosa chinensis* cv. 'Old Blush' leaflets could induce normal embryogenic calli with the 13.57 μM 2,4-D with 2.32 μM Kn, but petioles could not. With the extension in the culture time embryos gradually increased (Cai *et al.* 2022). Hence in the present study within the scope of *in vitro* culturing strategies, *Rosa pisiformis* (Christ.) D. Sosn. an endemic species under threat of extinction were micropropagated.

Materials and Methods

The present study was based on *Rosa pisiformis* (Christ.) D. Sosn. samples collected from Akçakale district of Gümüşhane province (Eastern Black Sea Region, Turkey). The shoots were defoliated and single nodes (2-5 cm) taken from actively shoots with length of 10-15 cm were used as the source of explants.

The initial explants were surface sterilized with antibacterial liquid soap by continuous shaking for 10 min, followed by washing under flowing faucet water until thoroughly rinsed. Then, they were decontaminated with fungicide Captan (3%) for 30 min. Further washing was continued under a sterile cabinet, where woody lower parts of explants were treated with mercury chlorite (1%) for 45 min and fragile upper leaves of explants were rinsed with sodium dichloroisocyanurate (NaCDD) for 30 min. Then, they were embedded in sterile distilled water for three times before being used as explants.

After the surface disinfection, the shoot explants with single node and 2-5 cm in length were transferred into 350 ml sterilized glass jars, containing 75 ml of two different media: MS and Driver and Kuniyuki walnut medium (DKW; Driver and Kuniyuki 1984) medium. Twenty eight shoot explants were taken per treatment replication. Then the number reduced to 21 in DKW, 22 in MS (1 : 0 : 1) and 17 in MS (2 : 0 : 2) hormone combination medium because of contamination at the end of the first subculture. The shoot proliferation was performed with different combinations of BAP (1 and 2 mg/l) and NAA (0.1 and 0.2 mg/l) for MS medium and BAP (0.6 mg/l) and NAA (0.01 mg/l) were considered for DKW medium. Both nutrient media were solidified with 7 g/l agar and sucrose was added at the rate of 30 g/l. The pH of the medium was adjusted to 5.8 ± 0.1 using 1 N NaOH or 1 N HCl in all the experiments. The medium was sterilized by autoclaving at 1.2 bars pressure and 121°C temperature for 20 min. All the *in vitro* cultured jars were maintained in a plant growth room at $26 \pm$

1°C under 16/8 hr (light/dark) photoperiod on aluminum growth shelves with a fluorescent light intensity of 2700 lux.

Subculturing was performed every 3 weeks culture period using MS medium supplemented with different concentrations of auxin (0.1 and 0.2 mg/l of NAA) and cytokinin (1 and 2 mg/l of BAP). Subculturing was also done every 3 weeks culture period using DKW medium supplemented with auxin (0.01 mg/l NAA) and cytokinin (0.6 mg/l BAP).

Proliferated shoots were inoculated on DKW medium supplemented with 1 - 5 mg/ IBA after dipped in a solution of an auxin for *in vitro* rooting. Healthy and well rooted plantlets from the previous stage were selected and taken from jars after 2 weeks, then the roots were fully washed with tap water. Plantlets were transplanted to the plastic pots with turf: perlite (3/1). The plastic pots were covered with polyethylene bags to sustain high humidity and then were placed in the growth chamber under a 16/8 hrs photoperiod, at a temperature of $25 \pm 3^\circ\text{C}$ and $75 \pm 10\%$ humidity for 2 weeks. The plants were watered with tap water every day. Then the polyethylene bags were detached and the plantlets were transferred to the green house. The shoot survival percentage was recorded after 1 month. All the experimental results were statistically analyzed by Duncan (Steel *et al.* 1997).

Results and Discussion

Pre-treatment of the shoot explants with single node and 2-5 cm in length with fungicidal and bacterial treatments has a significant impact on explant survival and microbial contamination. In *R. rugosa* leaves the best disinfection time was found to be 75% ethanol treatment for the 30s, and the mass fraction 0.1% HgCl_2 treatment 8 min. The method is used to treat the leaves, and the browning rate and mortality are relatively low (Chang *et al.* 2020).

In the present experiments treatment with antibacterial liquid soap containing a commercial content and fungicide Captan 3% and HgCl_2 1% together for lower woody parts of the shoot explant and also NaCDD 0.2% application for upper leaves of the shoot explant gave best results in the surface sterilization of *R. pisiformis*. The fungicide and bacterial pre-treatment provided high explant survival and low microbial contamination and was significantly superior to control (Fig. 1). Since there are two types of fungicides used in this treatment, systemic type and contact type, they provided effective control of microbial contamination (Fig. 1).

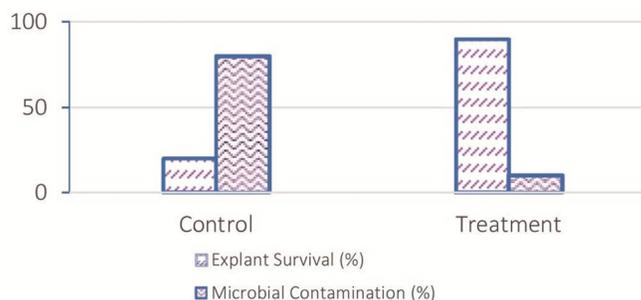


Fig. 1. Effect of pre-treatments on *in vitro* culture initiation in *Rosa pisiformis* (Christ.) D. Sosn. Control (distilled water); Treatment [(antibacterial liquid soap containing a commercial content and fungicide 3% + and HgCl_2 1% (for lower woody parts) + and NaCDD 0.2% (for upper leaves)].

Results also showed that shoot proliferation was significantly increased in DKW medium added with 0.6 mg/l BAP and 0.01 mg/l NAA. The best medium for shoot proliferation was DKW medium with 0.01 mg/l NAA: 0.6 mg/l BAP. While MS medium with 0.1 mg/l NAA: 1.0 mg/l BAP also gave the same number of shoots proliferation. The lowest leaf number was occurred in

MS. So, DKW medium was more succesful with the highest leaf formation. In this experiment, the use of BAP: NAA has an significant effect on both MS and DKW medium (Figs 2-3). Keeping the auxin : cytokinin ratio at all values vegetative propagations have a positive effect on *Rosa pisiformis* propagation (Table 1) .

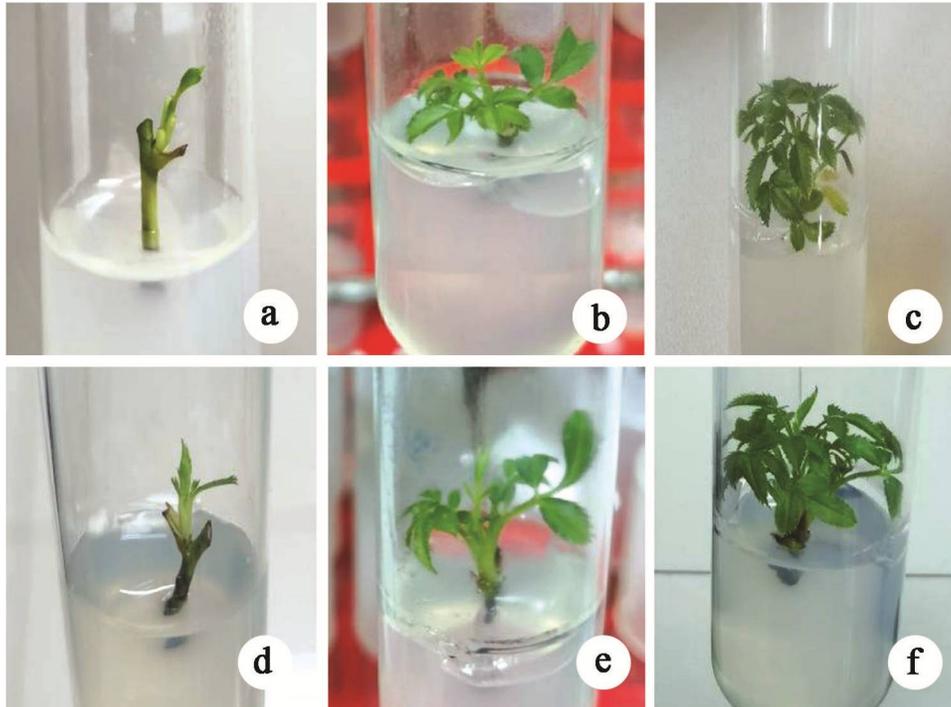


Fig. 2. Effects of interaction between BAP : NAA in MS on proliferation of *Rosa pisiformis* (Christ.) D. Sosn. nodal segments. (a) Explant establishment in MS with BAP 1.0 mg/l and NAA 0.1 mg/l (b) Multiple shoot formation with MS comprising BAP 1.0 mg/l + and NAA 0.1 mg/l after three weeks (c) Shoot proliferation on MS medium comprising BAP 1.0 mg/l and NAA 0.1 mg/l after two months of culture period (d) Explant establishment in MS comprising BAP 2.0 mg/l and NAA 0.2 mg/l (e) Multiple shoot formation with MS comprising BAP 2.0 mg/l and NAA 0.2 mg/l after three weeks (f) Multiple shoot formation on MS comprising BAP 2.0 mg/l and NAA 0.2 mg/l after two months of culture period.

Preliminary experiments were performed to determine the optimisation procedure for shoot propagation in DKW medium. The 0.6 mg/l BAP and 0.01 mg/l NAA combination were found to show the best growth rate of *Rosa pisiformis* explants grown *in vitro* in DKW medium (Fig. 3). The interaction effect of BAP : NAA appears to be important in shoot growth from nodal explants of *Rosa pisiformis* in both MS and DKW medium (Figs 2-3). The best medium to increase shoot number was observed with 0.02 NAA : 1 BAP in DKW medium and 0.1 NAA : 1.0 BAP in MS (Table 2).

According to results the impact of plant growth hormones on vegetative traits of *R. pisiformis* in proliferation stage was not significant on shoot numbers but significant on leaf numbers ($P \leq 0.05$) (Table 2). New leaf number was maximum on the DKW medium containing 0.6 mg/l BAP with 0.01 NAA mg/l (Fig. 3), whereas the maximum number of shoot was in the DKW with 0.6 mg/l BAP and 0.01 NAA mg/l, and MS with 1.0 mg/l BAP with 0.1 NAA mg/l (Fig. 2a,b,c).

Pahnekolayi *et al.* (2014) cultured *Rosa canina* node sections with 3 axillary buds in MS with BAP, NAA, and GA₃. The percentage of green leaves was highest in the application of 1.5 mg/l BAP followed by using 1.5 mg/l BAP + and 0.1 mg/l NAA. Considering the average shoot percentages, the *second highest* value was obtained in 2.0 mg/l BAP and 0.1 mg/l NAA. Markovic *et al.* (2021) stated that the highest iron chelate concentration leads to more shoots numbers in *Rosa canina*.

Table 1. Concentration ratios of NAA and cytokinin used to cultivate *Rosa pisiformis* vegetative proliferation on MS and DKW medium.

Medium	BAP (mg/l)	NAA (mg/l)	Ratio (BAP : NAA)
MS	1.0	0.1	1:0.1
MS	2.0	0.2	1:0.1
DKW	0.6	0.01	1:0,02

Table 2. Effects of different medium on some vegetative traits of *Rosa pisiformis* in proliferation stage.

Medium	BAP:NAA (mg/l)	Shoot number	Leaf number
MS	1:0.1	22.66 ^{ns}	208 ^b
MS	2:0.2	20 ^{ns}	102 ^{ac}
DKW	0.6:0.01	22.66 ^{ns}	222.66 ^b

ns: non- significant. Different letters indicate significant difference according to Duncan Test ($P \leq 0.05$) (Steel *et al.* 1997)

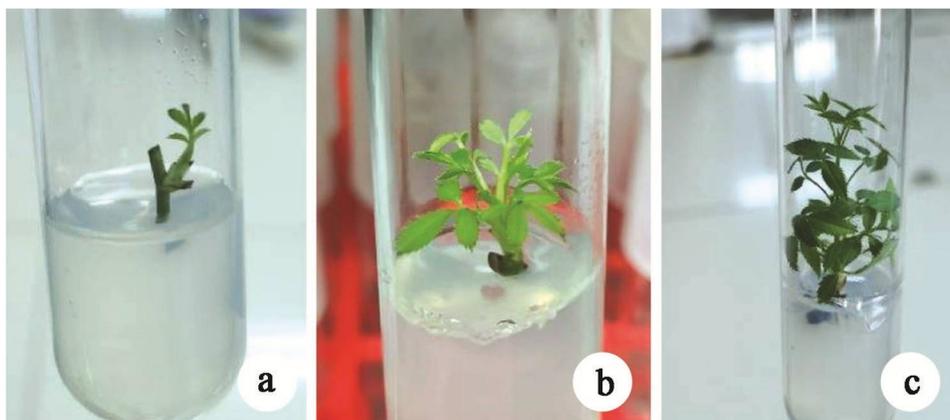


Fig. 3. Effect of 0.6 mg/l : 0.01 mg/l of NAA content in DKW medium on proliferation of *Rosa pisiformis* nodal segments. (a) Explant establishment (b) Multiple shoot formation after three weeks (c) Multiple shoot formation after two months.

Leaves obtained from axillary shoot proliferation medium were excised, sterilized and cultured in MS + 1.5 mg/l 6-BA + 3.0 mg/l NAA + 3% sucrose + 0.9% agar in *Rosa rugosa* tubb. to induce callus (Chang *et al.* 2020) with a rate of 75% compared with without hormone. MS + 2.0 mg/l 6-BA + 0.2 mg/l NAA + 3% sucrose + 0.9% agar, the callus diameter reached 2.49 cm after proliferation, the growth was strong, and the callus was highly available.

Rosa pisiformis leaf explants also occurred good callus in MS (1.5 mg/l BAP : 3.0 mg/l NAA). Leaf-derived light yellow fragile callus was subcultured on solid MS with different combinations of cytokinin and auxins (Table 3). Fresh weight and dry weight of callus increased gradually over 8 weeks.

Table 3. Concentration ratios of NAA and BAP used to cultivate *Rosa pisiformis* callus on MS.

Condition number	BAP (mg/l)	NAA (mg/l)	Ratio (BAP : NAA)
1	1.5	3.0	1:2
2	2.0	4.0	1:2
3	2.0	4.5	1:2.25

Table 4. Effects of different BAP : NAA concentrations on callus formation in rose cultivar in MS.

BAP (mg/l)	NAA (mg/l)	Fresh weight	Dry weight
1.5	3.0	0.428±0.364 ^{ns}	0.0298±0.015 ^{ns}
2.0	4.0	0.277±0.160 ^{ns}	0.0278±0.017 ^{ns}
2.0	4.5	0.294±0.169 ^{ns}	0.0253±0.013 ^{ns}

ns: non- significant. Different letters indicate significant difference according to Duncan Test ($P \leq 0.05$).

Present study showed that the differences between the treatments were not significant, however, the maximum callus formation of leaflets were significantly higher in the MS with 1.5 mg/l BAP with 3.0 mg/l NAA. As the concentration was 2.0 mg/l⁻¹ BAP with 4.0 mg/l NAA, a reduced callus growth rate was observed. The 1 : 2 ratio in cytokinin:auxin (mg/l) values was due to good callus (Fig. 4).



Fig. 4. Callus formation of leaflets of *R. pisiformis*.

Proliferated shoots were cultured in DKW medium with 1.0-5 mg/l of IBA. Rooting was performed in each of 44 randomly selected plants which previously transported to the auxin solution (Fig. 4) in one month period. *In vitro* rooting percentage was reported as 100%, root length and root number were reported as 20.4 ± 7.0 mm and 110.0 ± 0.6 respectively. Each treatment included 44 replications (Table 5).

Acclimatization of the rooted plantlets in turf and perlite 3:1 (v/v) was easily carried out at $\pm 25^{\circ}\text{C}$ and the relative humidity was 80%, then gradually reduced to 40% (Fig. 4). Shoots showed a good rooting response after about 14 days. After a few weeks of cultivation, the acclimatized plants were transplanted to the greenhouse.

Table 5. Effects of different medium on *in vitro* rooting of *R. pisiformis*.

Medium IBA (mg/l)	Shoot rooting (%)	Number of roots/shoot	Lenght per shoot (mm)
DKW 1.0	100.00	110.00	17.91 \pm 0.47 ^{a 0.000**}
DKW 3.0	40.90	18.00	5.54 \pm 1.24 ^b
DKW 5.0	20.45	11.00	2.55 \pm 0.83 ^b

Different letters indicate significant difference according to Duncan Test ($P \leq 0.01$).

In vitro rooting medium was MS (0.4 mg/l IBA, 0.5 mg/l of activated carbon, 3% sucrose and 0.9% agar) with the result of the rooting rate is 75.00%, the root system was long and the growth was robust in *R. rugosa* tunb. (Chang *et al.* 2020).



Fig. 4. *In vitro* rooted shoots of *R. pisiformis*. (a) Proliferated shoots with roots. (b). Roots bearing in rooting medium.



Fig. 5. *In vitro* rooted plantlets were habituated *ex vivo* conditions (a) 14 days after transplanted to the greenhouse (b).

Pre-sterilization of explants performed with mercury chlorite (1%) and sodium dichloroisocyanurate (NaCDD) showed *R. pisiformis* shoot proliferation was succeeded in DKW (0.6 mg/l BAP + 0.01 mg/l NAA) with the increase of shoot and leaf numbers. Good callus formation was obtained with 1.5 mg/l BAP : 3.0 mg/l NAA in MS medium and rooting was succeeded as 100% in DKW (1.0 mg/l of indole-3 butyric acid).

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