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Practical Factors Controlling *in vitro* Multiplication and Rooting in *Empetrum nigrum* var. *japonicum,* an Endangered Woody Species

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Abstract - The plant *Empetrum nigrum,* valued in the traditional system of medicine, is well known for its antibacterial, antifungal, and antioxidant properties. In the present work, the effect of removal of shoot apical meristem (SAM) on shoot proliferation was studied. It was observed that removal of SAM promoted shoot proliferation whereas intact tip resulted in higher survival percentage. Further, the effect of different concentrations of BA on above was also studied. During root formation the effect of light quality after treatment with IBA was investigated. For rooting, continuous red light without IBA resulted in maximum rooting percentage. The above factors when taken into consideration during micropropagation of this endangered plant can result in healthier plantlets. The results show that the species could be successfully conserved by *in vitro* propagation system.

Key words - *Empetrum nigrum,* Micropropagation, SAM, Shoot tip removal, Red light

Introduction

Empetrum nigrum var. *japonicum* (crowberry), a medicinal woody plant belongs to *Ericaceae* family, is one of the native berries of South Korea, especially on top of Mt. Halla (Han *et al*., 2010). *Empetrum* is a small genus of dwarf (10-30 cm in height) evergreen shrubs. The leaves and stems are used in Denaina medicine for diarrhea and stomach problems; they are boiled or soaked in hot water, and the strained liquid is consumed. Its extract exhibits diuretic and spasmolytic properties, aerial parts used in folk medicine to cure liver and kidney disease (Krasnov *et al*., 2000), certain components show antibacterial and fungicidal activity (McCutcheon *et al*., 1994). Several reports have indicated the antioxidant potential of this plant (Kahkonen *et al*., 1999; Kim *et al*., 2009; Kim *et al*., 2011). Although the *E. nigrum* var. *japonicum* is important as a medicinal material and one of Korean genetic resources, few reports have been focused on *in vitro* micropropagation of the species except the report by Han *et al*. (2010). Herein, we report the results for *in vitro* shoot multiplication and rooting of rooting-recalcitrant *E. nigrum* and it show that the possibility of improving the shoot proliferation by removing SAM and rooting by red light irradiation.

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Materials and Methods

Plant materials

Two years *in vitro* maintained *Empetrum nigrum* var. *japonicum* in hormone-free Woody plant medium (WPM; Lloyd and McCown, 1981) medium were used for the experiments. For rooting, 2 cm-length of shoots were used.

Shoot multiplication

For shoot proliferation, two types of plant material were used, shoot with intact apical meristem (ST) and shoot without apical meristem (N-ST). In both the BA concentration were varied as 0.2, 0.5, 1.0 mg/L with WPM as the basal medium.

Rooting

For rooting two sets of shoots were used. In one set the bottom of shoot was soaked into 1,000 mg/L IBA for 30 min, after that placed into hormone-free WPM containing sucrose

30 g/L and solidified with 2.5 g/L gelrite. The other set of shoots did not receive the IBA treatment. Thereafter both these sets of shoots received light treatments as indicated in Table 1.

For red irradiation, LED system (GF-320s) with, peak emission at 650 nm was used and fluorescent lamp was used for white light. The light intensity in all kinds of light treatment was 40 μmol $m^{-2}s^{-1}$ PPF. All the cultures were kept at 23±1℃ with 16/8-hr (day/night) photoperiod and cultured for 8 weeks.

Statistics

All experiments were repeated three times with ten replications. Statistical significance was determined using Duncan's multiple range test ($p \leq 0.05$) for a multi-comparison of means (SAS Institute, Cary, N.C.). Values marked with similar letters are not significantly different.

Results and Discussion

As previous report (Han *et al*., 2010) had shown that WPM medium was found to be the best medium for growth of *Empetrum nigrum* var. *japonicum,* in the present study it was used for shoot multiplication in combination with different concentrations of BA. It was observed that increasing BA concentration did not have any significant effect on shoot survival or proliferation. Further, the effect of removal of shoot apical meristem (SAM) from the explants was also studied. The SAM consists of a small group of undifferentiated and dividing cells with dense cytoplasm. Potten and Loeffler (1990) on the basis of several criteria suggested that the cells in the meristem can be classified as stem cells. These cells have the unique abilities to proliferate, to replace themselves when required, to give rise to a variety of differentiated cell types, and also can regenerate a new meristem if damaged (Sussex, 1952). Also, Reddy et al. (2004) reported that these

Table 1. Effect of apical meristem elimination from shoot explants on shoot multiplication from *in vitro* grown *Empetrum nigrum* var. *japonicum* after 8 weeks of culture

BA (mg/L)	Survival rate (%)		No of shoots (per explant)	
	ST	N-ST	ST	N-ST
0.0	93.3	83.3	1.0±0.09	2.7±0.6
0.2	100.0	93.5	1.0±0.10	2.8±0.5
0.5	100.0	93.3	1.0±0.21	3.0±0.4
1.0	100.0	92.1	1.0±0.13	3.0±0.4

ST Normal shoot with apical meristem
N-ST Apical meristem eliminated-shoot

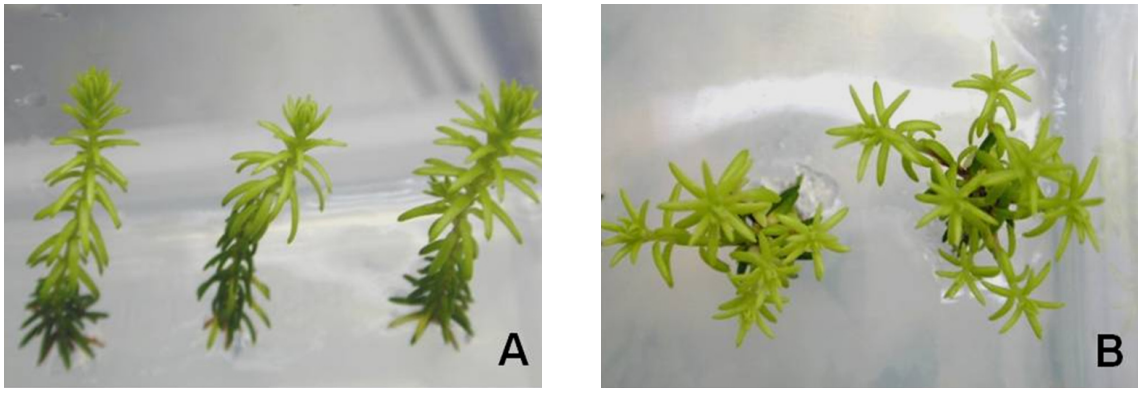


Fig. 1. Shoot multiplication from different type of explants after 8 weeks of culture in *Empetrum nigrum* var. *japonicum.* A: Normal shoots with apical meristem, B: Apical meristem eliminated-shoot.

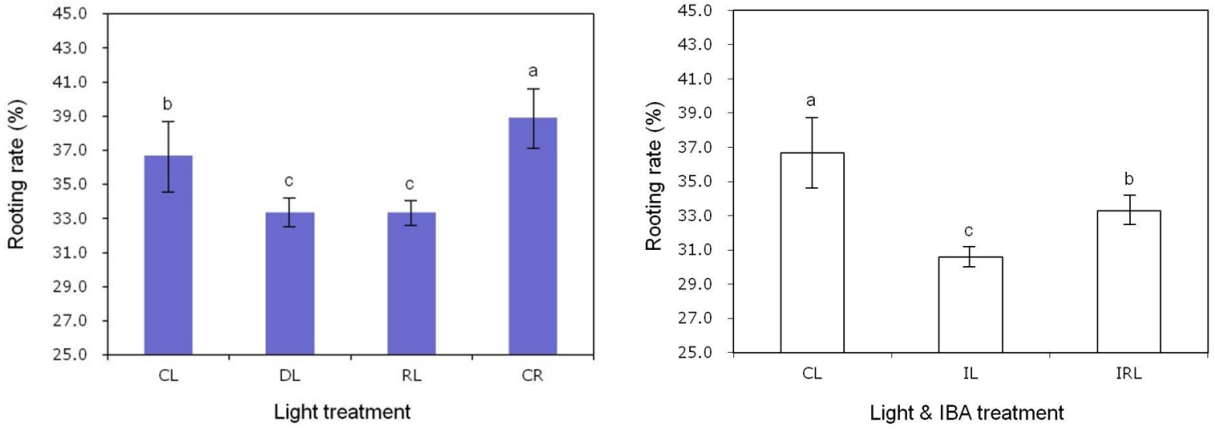
stem cells are responsible for initiation of lateral organs. In case of *Empetrum nigrum* var. *japonicum*, it was found that explants without SAM had a lower survival percentage (Table 1). Barton and Poethig (1993) while working on shoot apical meristem fewer mutants of *Arabidopsis* observed that conditions that promote shoot regeneration *in vitro* in the wild type could not result in normal shoot formation in the explants of mutant type. The latter only gave rise to abnormal leaves or shoots but its number was lesser than in wild type. They suggested that the gene stm-1 interferes with shoot formation *in vitro* but an organized SAM is not required for leaf and shoot formation both in culture and *in situ* (Barton and Poethig, 1993). Our results also indicate that in *Empetrum* presence of SAM is not essential for shoot formation *in vitro* (Fig. 1). In fact removal of shoot tip in the present genus resulted in higher shoot proliferation by suppression of apical dominance.

During rooting it was observed that in the absence of IBA highest rooting percentage was observed in shoots kept in continuous red light (39%) followed by shoots kept under continuous light for eight weeks (Fig. 2). This indicates that red light alone is sufficient for root development in *Empetrum*. Possibly other wavelengths of light exert a negative effect on root formation in this genus as rooting percentage reduced to

Table 2. Description of light and hormonal treatments for rooting in *E. nigrum* var. *japonicum*

Treatment	IBA	Week							
		1	2	3	4	5	6	7	8
E1: Light effect									
CL (Continuous light)	-	L	L	L	L	L	L	L	L
DL (Dark → light)	-	D	D	L	L	L	L	L	L
RL[z] (Red → light)	-	R	R	L	L	L	L	L	L
CR (Continuous red)	-	R	R	R	R	R	R	R	R
E2: Light & IBA effect									
CL (Continuous light)	-	L	L	L	L	L	L	L	L
IL[y] (IBA + light)	O	L	L	L	L	L	L	L	L
IRL (IBA+red →light)	O	R	R	L	L	L	L	L	L

[z]Red irradiation : LED system (GF-320s), peak emission : 650 nm.
[y]IBA treatment: Immersion into IBA 1,000mg L-1 solution for 30 min.



(A) (B)

Fig. 2. Effect of light (A) and IBA treatment (B) on rooting from *in vitro* shoots of *E. nigrum* var. *japonicum.* (Error bars represent for standard errors of mean, and the letter on the error bars represent for significance by Duncan's multiple range test, $p \leq 0.05$).

37% in white light. Further, it was also seen that providing red light for only first two weeks followed by continuous white light resulted in poor rooting. Taiz and Zeiger (1991) reported that growth and development in plants is influenced by the quality, intensity and duration of light. Similarly, growth, morphology and differentiation of *in vitro* plantlets are also affected by light (Economou and Read, 1987). Red light has been shown to significantly enhance stem elongation in *Pelargonium* plantlets (Appelgren, 1991); higher root to shoot ratio in potato plantlets (Aksenova *et al*., 1994) and affect morphology rather than growth of potato plantlets when used as main light source (Miyashita *et al*., 1995).

However, in the present study when the shoots are pretreated with IBA, root formation was considerably reduced (31%) even in presence of continuous light (Fig. 2). When after IBA treatment, the shoots were kept in red light for two weeks and then transferred to white light, the rooting percentage improved a little (33%). Any plant under normal environmental conditions can proceed via either of two developmental pathways: photomorphogenesis in the light and skotomorphogenesis in the dark (von Arnim and Deng, 1996). The choice that a plant makes between these two pathways is crucial for its development and also ensures survival in its environment. To select the appropriate pathway, plants possess several photoreceptors to detect the different qualities and quantities of light (Fankhauser and Chory, 1997). Several physiological experiments have indicated that auxin is the major plant hormone closely connected with light signal transduction (Neff *et al*., 1999; Steindler *et al*., 1999). In addition to hypocotyl elongation, auxin affects numerous aspects of plant growth and development such as gravitropism, lateral root differentiation and apical dominance (Estelle and Klee, 1994; Hobbie, 1998). Auxin has different effects on different tissues: it stimulates cell elongation in stems and hypocotyls, whereas in roots it stimulates cell division for lateral root formation (Estelle and Klee, 1994; Hobbie, 1998). However, in *Empetrum* auxin pre-treatment for 30 min has a negative influence on rooting. This is similar to the findings of Nakazawa *et al*. (2001) in *Arabidopsis*. They observed that the gene product of DFL1 gene (an auxin responsive gene) in *Arabidopsis* inhibits

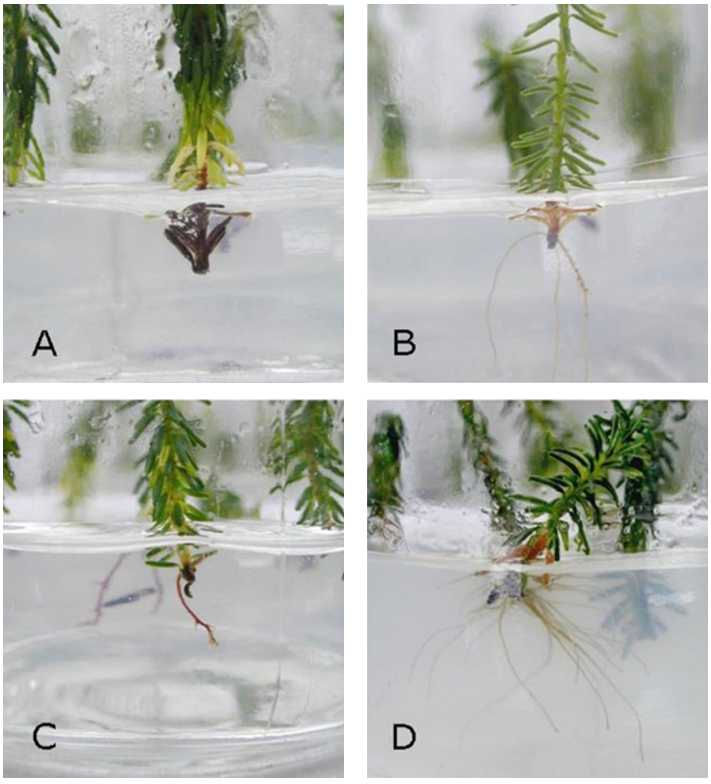


Fig. 3. Effect of light quality on rooting in *E. nigrum* var. *japonicum* after 8 weeks of culture. A: IBA + light (IL), B: IBA + red to light (IRL), C: Dark to light (DL), D: Continuous red irradiation.

lateral root cell differentiation in light though it has no effect on primary root formation. Since the lateral roots emerge from the primary root by a series of divisions in the pericycle cells (Malamy and Benfey, 1997) probably in *Empetrum* auxin exposure has a negative effect on this activity. The positive effect of red light is seen even in presence of IBA indicating that in this genus the quality of light can overcome the inhibition by auxin.

Thus the present study indicates that SAM and light quality can affect *in vitro* propagation of *Empetrum nigrum* var. *japonicum*. This information can be used for increasing the efficiency of micropropagation of this endangered medicinal plant.

Acknowledgement

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