

Sexual and Vegetative Propagation of *Hypericum empetrifolium* Willd. subsp. *empetrifolium*

Anastasia AKOUMIANAKI-IOANNIDOU^{1*}, Ekaterini GERASIMIDOU¹,
Alexandra SALTA¹, Ioannis ROUSSIS², Dimitrios BILALIS²

¹Agricultural University of Athens, Department of Crop Science, Laboratory of Floriculture and Landscape Architecture, 75 Iera Odos Str., 118 55 Athens, Greece; akouman@aua.gr (*corresponding author); katerina.gerasimidou@gmail.com; alexandrasalta@yahoo.gr

²Agricultural University of Athens, Department of Crop Science, Laboratory of Agronomy, 75 Iera Odos Str., 118 55 Athens, Greece; iroussis01@gmail.com; bilalisdimitrios@gmail.com

Abstract

Hypericum empetrifolium Willd. subsp. *empetrifolium* is an evergreen small shrub with small elongated decorative leaves and small yellow flowers in inflorescences, characterized for several pharmaceutical properties. In the present study, a first approach on the sexual and asexual propagation of this species was performed. Seeds, subjected to different types of pre-treatments [soaked in tap water; 50, 100 or 150 mg L⁻¹ GA₃ for 30 min and no treatment (control)], cultured for germination in petri dishes at 5, 10, 15, 20, 25, and 30 °C. Seed germination was only affected by temperature and the best result was obtained at 15 °C (71.2%). A significant interaction was found between pre-treatments and incubation temperature with the highest germination percentage (82%) occurred when the seeds soaked in 100 mg L⁻¹ GA₃ solution for 30 min and incubated at 20 °C constant temperature. Moreover, the germination speed was fastest from 20 to 25 °C (T₅₀= 9.84 and 9.56 days for 20 and 25 °C, respectively). For asexual propagation, apical stem cuttings were taken at four different periods (4 seasons) and treated with IBA at concentrations of 0, 1000, 2000 and 3000 mg L⁻¹. The cuttings were planted in a peat/perlite mixture 1:1 v/v in plastic square plug trays in order to study the rooting percentage. Winter was the most appropriate season for cuttings collection (100% rooting percentage) and dipping in 1000 or 2000 mg L⁻¹ IBA (72% and 73%, respectively) was the best rooting hormone treatment.

Keywords: indole-3-butyric acid (IBA); medicinal plant; ornamental value; seed germination; stem cutting

Introduction

Hypericum empetrifolium Willd. subsp. *empetrifolium* (Hypericaceae) is a small evergreen shrub, widespread at low altitudes in the Aegean area, the south part of the Greek mainland and the coastal area of western Turkey (Trovato *et al.*, 2001). Its stem is up to 50 cm tall, erect or prostrate with strict branching from the base. Leaves are 2-12 mm, short, narrow, needle-like in whorls of three and hairless. Deep yellow flowers are numerous and densely black-dotted in elongated panicles or simple cymes that form from April to June. The fruit is an ovate capsule with oblique vesicles (Davis, 1988). In Greece, *H. empetrifolium* subsp. *empetrifolium* is traditionally used as a medicinal plant with high antioxidant and antibacterial activity, while decoctions of the flowers are taken internally as an anthelmintic and diuretic and used externally as a wash to speed healing of wounds, scalds, and herpes (Petrakis *et al.*, 2005). Several

phytochemical investigations on this species have been described the presence of naphthodianthrone and flavonoids in crude extracts of the flowers (Kitanov, 2001) as well as the composition of the essential oil (Petrakis *et al.*, 2005).

Apart from medicinal value, *H. empetrifolium* subsp. *empetrifolium* has potential value use as a landscape plant because of its yellow and black-dotted flowers forming a nice contrast to the dark green foliage. In addition, this plant does not lose its leaves in the winter and retains this wonderful ornamental value even in the cold season. This plant species could be used in Mediterranean parks, gardens and archaeological sites contributing to the biodiversity enrichment and reflecting the traditional character of the area (Diekelmann and Schuster, 2002; Papafotiou *et al.*, 2017).

Seed germination consists a critical stage of the plant life cycle and usually controls the plant population dynamics

with significant practical consequences (Çirak, 2007). In spermatophytes, it is a complex trait which is influenced by many genes and environmental factors (Carta *et al.*, 2016). The seeds of 59 *Hypericum* species have been recorded as orthodox, indicating that the life cycle of these species in the field may be related with deep seed dormancy (Sanchez-Coronado *et al.*, 2015). Indeed, it has been found that the germination capacity of *Hypericum* species is very low mainly due to the prolonged seed dormancy (Carta *et al.*, 2016). The low seed germination in *H. perforatum* (Campbell, 1985) and *H. aviculariifolium* (Çirak *et al.*, 2007) is caused by a chemical inhibitor in the capsule. Plant growth regulators such as GA₃ (gibberellic acid) and IAA (indole-3-acetic acid) have been recommended to break seed dormancy and enhance seed germination (Çirak, 2007). Çirak *et al.* (2011) found that GA₃ increased germination rate of *H. triquetrifolium* significantly as compared to control; 68% germination for seed treated with 100 mg L⁻¹ GA₃ compared to approximately 30% for untreated seeds. Other factors, reported to affect the seed germination of several *Hypericum* species, are light and fluctuating temperatures which are common requirements for small-sized seeds (Thompson *et al.*, 2001). Carta *et al.* (2016) found the optimum temperatures for germination of *H. elodes* to be alternating 30/20 °C with a 12-h daily photoperiod, whereas Bertelle *et al.* (2004) suggested that a temperature of 20 °C and 30 °C is the recommended for germination in *H. perforatum* and *H. brasiliense* seeds, respectively.

The vegetative or asexual propagation by stem cuttings is a simple and easily applied method of propagation, while it offers productions of true-to-type plants in a short time period and availability of superior individuals for large scale commercial plantation with quick productive gains (Shekhawat and Manokari, 2016). However, for each plant species, it is necessary to know the appropriate concentration of the rooting hormone and cutting collection period. Auxins are commonly used in cutting propagation as root-promoting chemicals (Lu *et al.*, 2008). Nevertheless, it has to be noted that, the effect of exogenous application of auxins may vary from ineffective to promotive, or even inhibitory for the rooting of cuttings, depending on the endogenous level of growth-regulating substances or the tissue sensitivity (Akoumianaki-Ioannidou *et al.*, 2016). This results in the definition of the optimal concentrations of auxins for the rooting of each species. Regarding the time of cuttings collections, it plays an important role in rooting success and development of cuttings, because of the changes in the endogenous plant growth regulators or carbohydrate conditions of cuttings as well as the conditions in the nursery growing environment (Klein *et al.*, 2000).

A survey of the literature revealed than no study on sexual or asexual propagation of *H. empetrifolium* subsp. *empetrifolium* has been undertaken. The purpose of this study was to investigate seed germination and asexual propagation of this species, in order to use as an ornamental plant in urban and suburban areas as well as a medicinal plant with potential usage in the pharmaceutical industry.

Materials and Methods

Experiment 1: Seed germination of H. empetrifolium subsp. empetrifolium

Dried seeds of *H. empetrifolium* subsp. *empetrifolium* were collected in August 2015 from selected mature plants in the region of 'Panagia Orphani' (36°15'21.8"N, 22°55'45.4"E, altitude 102 m) in Kythira island, Greece and were stored in plastic bags at room temperature conditions (about 21 °C) under darkness. A germination experiment was carried out twice; in June and August 2016. Seeds were surface sterilized by soaking in 15% chlorine dioxide solution for 10 min and then rinsed for 20 min with distilled deionized water. After the rinsing, seeds received various pre-treatments [soaked in tap water; 50, 100 or 150 mg L⁻¹ GA₃ for 30 min and no treatment (control)] and then they were placed in 100 × 15 mm petri dishes, on two sheets of filter paper moistened with 3 ml distilled water at six constant temperatures (5, 10, 15, 20, 25 and 30 °C). Incubation of seeds took place with a 16-h light/ 8-h dark photoperiod. Cool white fluorescent tubes provided lighting with an irradiance of 37.5 μmol m⁻² s⁻¹. Each petri dish contained 10 seeds and represented one replication or experimental unit. Five replications per temperature and treatment were used. Germination percentages were evaluated every 2 days for 36 days and a seed was considered germinated when the radicle was longer than 1 mm. T₅₀ was also calculated according to Coolbear *et al.* (1980). The T₅₀ was not estimated when the final germination percentage was < 5%.

Experiment 2: Vegetative propagation of H. empetrifolium subsp. empetrifolium

Regarding asexual propagation, apical stem cuttings, 8-10 cm long, were excised from a population of native adult plants grown in the region of 'Diomedes Botanic Garden' (38° 00' 39.2" N, 23° 38' 11. 32" E, altitude 157 m) in Haidari, a western suburb of Athens, Greece, in February, April, August and October indicative of the seasons spring, summer and autumn and winter, respectively. Two experiments were conducted during 2016 and 2017, but due to the similarity of the results, only data of one year are presented. In winter, stem cuttings were collected from the new growth of the plant, which was started to sprout. During the spring and summer, cuttings were excised from non-flowering shoots. The stem cuttings collected in autumn were more lignified due to the stop of shoot growth. All leaves and axillary shoots were removed from the basal half of the stem cuttings. The bases of the stem cuttings (around 1.5 cm of the bottom) were immersed in IBA ethanol-water (1:1, v/v) solutions, at different concentrations: 0 (control), 1000, 2000 and 3000 mg L⁻¹, for 1 min, and then placed for rooting in plastic square plug trays (cell dimensions: 5.0×5.0×5.0 cm) containing a substrate of peat and perlite (1:1, v/v) in a mist system (15 s spraying every 15 min from May to September or every 30 min from October to April; substrate temperature 22 °C maintained by thermostatically controlled electric heating cable) for eight weeks. Three replications with ten cuttings were used in each treatment.

Rooting percentages were determined every two weeks by checking cutting resistance in pulling and root emergence through the hole at the bottom of each planting cell.

Statistical analysis

For the statistical analysis, JMP 8 statistical software (SAS Institute Inc., Cary, USA) was used. All the experiments were conducted according to completely randomized design (CRD). The significance of the results was tested by one or two-way ANOVA and differences between means were separated using the *LSD* test. All comparisons were made at the 5% level of significance ($p \leq 0.05$). Finally, the data on percentage were statistically analyzed after arcsine transformation.

Results and Discussion

Experiment 1: Seed germination of *H. empetrifolium* subsp. *empetrifolium*

The effect of pre-treatments on seed germination was found not to be statistically significant (Table 1). However, GA₃ (regardless of the concentration) increased the percentage of germinated seeds in comparison with the controls and the “tap water-treated” seeds at 10 °C.

Maintenance of dormancy is a consequence of high abscisic acid (ABA) content in mature seed and dormancy release has a strongly correlation with the reduction of ABA content. Gibberellins reduce the effects of ABA and overcome several types of seed dormancy, including physiological dormancy, photodormancy and thermodormancy acting as a substitute for low temperatures, long days or red light, respectively (Salisbury and Ross, 1992). The application of gibberellic acid (GA₃) to plants is known to modify the effect of cytokinins on transport across membranes and is thus able to initiate the biochemical processes necessary for seed germination. The cytokinin possibly penetrates the testa and neutralizes the inhibitors present in the embryo, enabling the embryo to rupture the seed coats (Chen *et al.*, 2008). In general, the application of GA₃ promotes germination of several *Hypericum* species such as *H. perforatum* and *H. pruinatum* indicating the presence of physiological dormancy related to partially dormant embryo (Cırak *et al.*, 2007).

The temperature at which seeds were incubated after pre-soaking treatments significantly affected final germination percentages (Table 1). The optimum temperature for seed germination was 15 °C at which the maximum germination percentage (76%) was achieved without any pretreatment indicating that there was no dormancy in seeds after their storage for 10-12 months.

Table 1. The effect of pre-treatments and temperature on seed germination and germination speed (T_{50}) of *Hypericum empetrifolium* subsp. *empetrifolium*

| Treatment | Temperature (°C) | Germination (%) ± SE | T_{50} |
|--|------------------|---------------------------------|---------------------------------|
| Untreated (Control) | 5 | 0.0 ± 0.0 | - |
| | 10 | 20.0 ± 8.4 | 26.8 |
| | 15 | 76.0 ± 5.1 | 15.1 |
| | 20 | 62.0 ± 9.7 | 9.6 |
| | 25 | 34.0 ± 4.0 | 12.0 |
| | 30 | 0.0 ± 0.0 | - |
| Tap Water | 5 | 0.0 ± 0.0 | - |
| | 10 | 14.0 ± 7.4 | 23.5 |
| | 15 | 70.0 ± 5.5 | 15.4 |
| | 20 | 80.0 ± 7.1 | 9.6 |
| | 25 | 28.0 ± 7.3 | 11.2 |
| | 30 | 0.0 ± 0.0 | - |
| GA ₃ 50 mg L ⁻¹ | 5 | 0.0 ± 0.0 | - |
| | 10 | 46.0 ± 12.9 | 23.3 |
| | 15 | 72.0 ± 4.9 | 14.4 |
| | 20 | 64.0 ± 5.1 | 9.6 |
| | 25 | 36.0 ± 8.1 | 8.4 |
| | 30 | 4.0 ± 4.0 | - |
| GA ₃ 100 mg L ⁻¹ | 5 | 0.0 ± 0.0 | - |
| | 10 | 40.0 ± 7.1 | 23.2 |
| | 15 | 66.0 ± 2.4 | 13.8 |
| | 20 | 82.0 ± 5.8 | 9.4 |
| | 25 | 44.0 ± 10.3 | 9.2 |
| | 30 | 0.0 ± 0.0 | - |
| GA ₃ 150 mg L ⁻¹ | 5 | 0.0 ± 0.0 | - |
| | 10 | 50.0 ± 12.2 | 23.4 |
| | 15 | 72.0 ± 3.7 | 15.0 |
| | 20 | 68.0 ± 8.6 | 9.6 |
| | 25 | 30.0 ± 5.5 | 8.4 |
| | 30 | 0.0 ± 0.0 | - |
| $F_{pre-treatment}$ | | ns | *(LSD _{5%} = 1.026) |
| $F_{temperature}$ | | *** (LSD _{5%} = 4.264) | *** (LSD _{5%} = 4.019) |
| $F_{pre-treatment \times temperature}$ | | ** (LSD _{5%} = 17.396) | ns |

F-test ratios are from ANOVA. Significant at *, ** and *** indicate significance at $p \leq 0.05$, 0.01 and 0.001, respectively and ns: not significant. The *LSD* test ($p \leq 0.05$) values for pre-treatment and temperature are also presented. SE: Standard Error.

In other species of genus *Hypericum*, namely *H. orientale*, *H. perforatum*, *H. pruinatum*, *H. origanifolium*, *H. triquetrifolium*, *H. heterophyllum*, the recommended temperature for seed germination was set at 20 °C (Çirak, 2007; Çirak et al., 2011).

In a previous study, it was found that germination of *Hypericum elodes* was greatly promoted in seeds subjected to diurnal alternating temperatures of 20/10 and 25/15 °C (Carta et al., 2016). However, all these researchers suggested cold stratification at 5 °C cold for 12 weeks, and/ or light and exogenously applied GA₃, KNO₃, hot water and tap water for breaking the seed dormancy of aforesaid *Hypericum* species (Çirak, 2007; Çirak et al., 2011; Carta et al., 2016).

According to Carta et al. (2016), the environment during seed maturation affects dormancy degree in *Hypericum* species. Several environmental factors, including light, moisture, temperature and nutrients, as well as the age of the mother plant during seed growth and maturation and the position of the seeds on the plant can lead to variations in germination behaviour among seeds of a species (Guterman, 2000).

A significant interaction was found between pre-soaking treatments and incubation temperature. The highest germination percentage (82%) occurred when the seeds soaked in 100 mg L⁻¹ GA₃ solution for 30 min and incubated at 20 °C constant temperature. This observation indicates that GA₃ can be used for the breakage of seed dormancy at supra-optimal high temperatures, as has been shown in lettuce (Dong et al., 2012).

The incubation temperature significantly affected the germination rate, as expressed by T₅₀ values. Generally, the germination speed (T₅₀) was enhanced by the increase of temperature. It was not possible to calculate T₅₀ for 5 °C and 30 °C due to the low final germination (<5%) at these temperatures. The germination speed for the range of the tested constant temperatures allowed an optimal range to be identified from 20 to 25 °C (9.84 and 9.56 for 20 and 25 °C, respectively). Poor germination at very low and high temperatures in legume species such as *Vigna unguiculata* may indicate that little or no germination will take place during winter or mid-summer and could act as a protection mechanism against excessive seedling mortality (Balkaya, 2004). Regarding the effect of pre-soaking treatments on germination speed, the results of this experiment demonstrated that exogenously applied GA₃ at 100 mg L⁻¹ shows promise as a practical method of improving the germination percentage, rate, and uniformity of *H. empetrifolium* subsp. *empetrifolium*.

Experiment 2: Vegetative propagation of *H. empetrifolium* subsp. *empetrifolium*

Root initiation and development in stem cuttings is controlled by a complex of ecophysiological and biochemical processes and morphological factors, as well (Uniyal et al., 1993). There is an overpowering evidence that auxins have been shown to improve overall rooting percentages, hasten root initiation and increase the number and quality of roots, which can be either naturally occurring within the plant (endogenous) or applied to the plant

(exogenous) throughout vegetative propagation (Blythe et al., 2007; Hartmann et al., 2011). Synthetic auxins such as indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA) are commonly used to promote root development in the vegetative propagation. Auxins promote the starch hydrolysis as well as the mobilization of sugars and nutrients at the base of the cuttings during the regeneration of adventitious roots (Nanda et al., 1974). The rooting success depends upon the proper balance between nutrient and auxin levels in cuttings.

According to the analysis of variance, the different IBA concentrations had also significant effect (F = 3.356, p = 0.024) on rooting. The higher average rooting percentages were achieved at 1000 and 2000 mg L⁻¹ IBA (72% and 73%, respectively; Fig. 1). The untreated stem cuttings had shown the least rooting performance (60%). Thetford and Miller (2002) conducted an experiment to investigate the influence of IBA and NAA on rooting of stem cuttings of *Hypericum reducium* and found that cuttings can root in high percentages up to 70% even without the use of exogenous auxins. The application of auxin is known to intensify root-forming process in stem cuttings. Usually polysaccharide hydrolysis is activated under the effect of applied IBA, and as a result, the content of physiologically active sugar increased providing materials and energy for meristematic tissues and later for root primordia and roots (Leakey et al., 1982).

Although auxins have been found to catalyze enzymatic reactions and thus increase the rate and quality of root production, in high concentrations they can have the opposite effect and retard or inhibit the formation of roots (Puri and Verma, 1996). In general, the use of auxins stimulates rooting of different propagating materials, but the concentration to be used for this purpose varies with the species, the maturity level of the propagating material, the environment as well as the mode of application of the plant growth regulators to the plants (Leakey et al., 1982; Akoumianaki-Ioannidou et al., 2016).

Rooting of the stem cuttings was also significantly affected by the collection season (F = 66.203, p < 0.001; Fig. 2) and is attributable to morphological and physiological characteristics of the stock plant at time of cutting collection (Hartmann et al., 2011). Highest rooting rates were achieved in winter (100%) in all treatments regardless of IBA application. In autumn, the average rate of rooting percentage was 77.5% with significant difference compared to the winter. In summer, rooting was relatively limited (61%) and in spring much lower (28%). The superiority of winter may be related to the fact that cuttings of several species taken in February contain higher level of sugars and total carbohydrate content and have higher eroxidase enzyme activity, which are found to have positive relationships with rooting response (Veierskov et al., 1982; Bhardwaj and Mishra, 2005). Moreover, the new vegetation of *H. empetrifolium* subsp. *empetrifolium*, which sprouts in the late of the winter, has as a result, the cuttings collected during this period, may be richer in endogenous auxins produced at the active apex of the young shoot, and transported basipetally to the cut surface in sufficient amounts to act as trigger (Nordström and Eliasson, 1991).

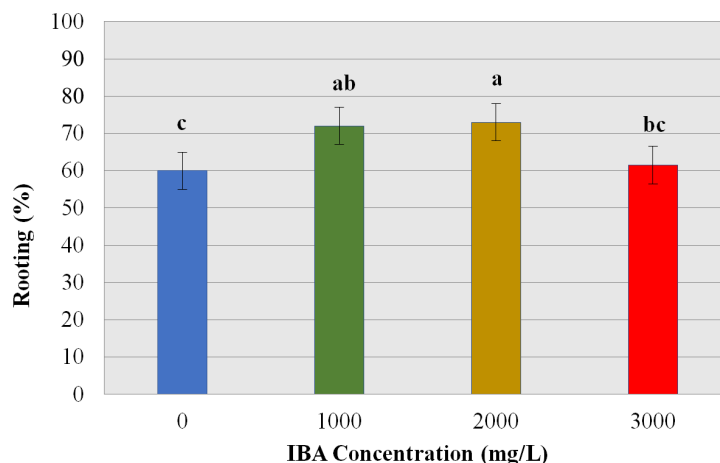


Fig. 1. The effect of IBA concentration on rooting percentage of *Hypericum empetrifolium* subsp. *empetrifolium* stem cuttings, regardless of season collection. Vertical bars and different lower-case letters denote standard errors of the means and statistically significant differences ($p \leq 0.05$), respectively

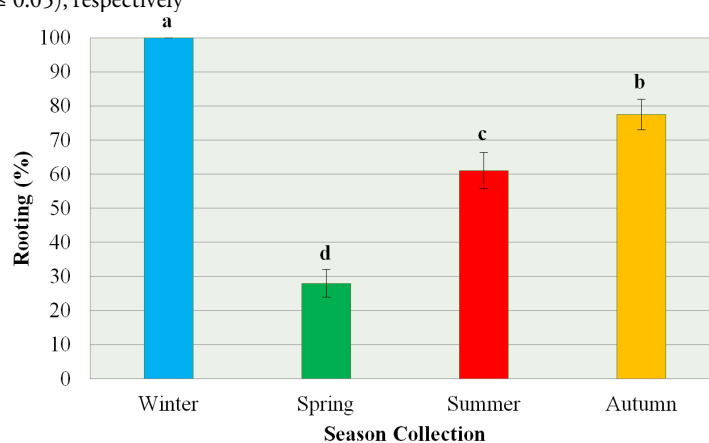


Fig. 2. The effect of season collection on rooting percentage of *Hypericum empetrifolium* subsp. *empetrifolium* stem cuttings. Vertical bars and different lower-case letters denote standard errors of the means and statistically significant differences ($p \leq 0.05$), respectively

Contrariwise, in the literature, it is referred that within the genus *Hypericum*, the late-summer softwood cuttings from tips of current growth can root easily (Thetford and Miller, 2002). Ambient temperature is rather unlikely affect rooting, as the temperature in the mist system was constant. Finally, in the vegetative propagation experiment, interaction between IBA and collection season was not found ($F = 1.650$, $p = 0.120$).

Conclusions

In conclusion, data of the present study indicated that the seeds of *H. empetrifolium* subsp. *empetrifolium* did not exhibit dormancy. However, exogenously applied GA_3 at 100 mg L^{-1} can be used, as a practical method, to improve the germination percentage, rate, and uniformity of this species. Regarding vegetative propagation, rooting of stem cuttings was affected both by collection season and rooting hormone treatment. Winter was the most appropriate season for cuttings collection and dipping in 1000 or 2000 mg L^{-1} IBA was the best rooting hormone treatment. These

results should provide a basis from which efforts may be made toward the advancement of vegetative propagation of *H. empetrifolium* subsp. *empetrifolium* by stem cuttings.

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