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# The effect of different plant hormones (PGRs) on multiple shoots of *Hypericum retusum* Aucher

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**Abstract**

Use of *Hypericum* species have increased in the past few years due to the antidepressant and antiviral activities found in extracts of those plants. As a result of its potential as a pharmaceutical, a new system was developed for *in vitro* culture of this species. The goal of this investigation was to produce multiple shoot via *in vitro* techniques for *Hypericum retusum* Aucher. *In vitro* germination of the seeds was standardized on Murashige and Skoog (MS) hormone-free medium. Cultures were initiated from shoots inoculated onto MS medium supplemented individually with nine different concentrations of 6-Benzylaminopurine (BAP) and Kinetin (Kn). The highest number of shoots was obtained on medium supplemented with 0.5 mg $l^{-1}$ BAP (64.25 shoot/explant). Out of all the investigated concentrations of Kn, the best result was obtained on medium supplemented with 1.5 mg $l^{-1}$ Kn (27.87 shoot/explant). In addition, shoots were cultured separately on the media containing BAP (0.5 $mgl^{-1}$) and Kn (1.5 $mgl^{-1}$) combined with three different auxins (0.25 $mgl^{-1}$ IAA, IBA, NAA). In view of number and length of shoot the best result was obtained on the medium supplemented with 0.5 mg $l^{-1}$ BAP + 0.25 mg $l^{-1}$ IBA (54.12 shoot/explant, 3.36 length of shoot). In the presented study, the use of the BAP alone was the most efficient for shoot propagation. Rooted plantlets were acclimatized and successfully transferred to soil.

**Keywords:** *Hypericum retusum* Aucher, Plant Hormones, The multiple shoot induction.

**Abbreviations:** BAP_6-Benzylaminopurine; IAA_Indole-3 acetic acid; IBA_Indole-3 butyric acid; Kn_Kinetin (6-Furfurylaminopurine); NAA_α-Naphthelene acetic acid

## Introduction

The *Hypericum* genus, a member of the Clusiaceae family, is represented in Turkey by 89 species, 43 of which are endemic (Baytop, 1984). This genus is widespread in Turkey and the most abundant and well-known species is *Hypericum perforatum* L. (Davis,1988). It is herbaceous perennial plant that has received considerable interest worldwide (Çırak et al., 2006). The *Hypericum* species contains a number of biologically active detectable compounds, naphtodianthrones (hypericin and pseudohypericin), phloroglucinols (hyperforin and adhyperforin), flavonoids, procyanidins, tannins, essential oils, amino acids, phenylpropanoids, xanthones and other water-soluble components (Greeson et al., 2001). Pharmacological experiments have shown that this species has antiulcerogenic, anticiceptive, anti-inflammatory, antitumor, antimicrobial and antioxidant activities ( Apaydın et al., 1999; Sökmen et al., 1999; Ozturk et al., 2002; Conforti et al., 2007).

*In vitro* systems have been reported as an effective tool for obtaining genetically uniform plants, which can be the source for less variable pharmaceutical preparations (Santarem and Astarita, 2003). Furthermore, plant tissue and cell cultures are also important tools which allow extensive manipulation of the biosynthesis of secondary compounds and yield a higher productivity compared to that of intact plants (Kirakosyan et al., 2001). Plant regeneration of the *Hypericum* species has been achieved by using as explants the whole seedling or their excised parts (Ćellárová et al., 1992), hypocotyl sections (Murch et al., 2000; Murch et al., 2002; Zobayed et al. 2004), leaves (Pretto and Santarém, 2000) and leaf discs and stem segments (Ayan et al., 2005), adventitious roots (Goel et al.,2009) using various types and concentrations of cytokinins and auxins.

Recently, many studies have been carried out to increase hypericin and pseudohypericin production within the *Hypericum* species (Kirakosyan et al., 2000; Walker et al., 2002; Gadzovska et al., 2005). Ayan and Çırak (2008), reported that there is a close relationship between the hypericin and the pseudohypericin contents of plant tissues and growth stages in

*H. triquetrifolium*. The effects of UV-C radiation on the increase of hypericin in *H. triquetrifolium* Turra were investigated by using *in vitro* techniques (Namli et al., 2009). Özen et al. (2004) reported that the fatty acid compositions of flowering tops of *H. perforatum* L. and *H. retusum* Aucher were analyzed. The major components were C16:0 (24.87%), C18:3 *n*-3 (21.94%), 3-OH-C18:0 (18.46%) and 3-OH-C14:0 (14.22%) for *H. Perforatum* L. and 3-OH-C14:0 (28.29%), C18:0 (16.47%) and C16:0 (14.17%) for *H. Retusum* Aucher.

Micropropagation is an advanced vegetative propagation techniques for producing a large number of genetically uniform and pathogen-free transplants in a limited time and space (Zobayed and Saxena, 2003). Each plant species propagated *in vitro* has requirements different plant growth regulators and their concentrations. Also, optimization of culture conditions and media is significant for micropropagation studies.

To the best of our knowledge, no report is available as regards *in vitro* propagation of *H. retusum* Aucher described by Robson (1967). Therefore, the objective of the presented investigation was to develop a procedure for the multiple shoot induction. Moreover, the most important point is that in vitro propagation of *H. retusum* is a key step (transition stage) to have adequate materials in order to be extracted and have the medical component available to be used in further studies.

## Materials and methods

### *Plant Material*

*Hypericum retusum* Aucher were collected from Diyarbakır (Turkey) in June 2007. Voucher specimens are kept at the Herbarium of Dicle University, Faculty of Science and Art.The seeds were used as explants to initiate *in vitro* cultures. They were surface-sterilized by immersion in a 5% (w/v) commercial bleach solution (NaOCI) for 10 min. after pre-sterilization processes, which included washing with tap water for 5–10 min followed by rinsing with 70% (w/v) ethanol for 30 s. Then, the seeds were rinsed with sterilized water 5 times for 5 min to remove the NaOCI. After which, they were dried by using sterile filter paper before inoculating them into basic MS culture medium (Murashige and Skoog, 1962). The seeds were germinated in a hormon-free medium under *in vitro* conditions. All the shoot tips used in the subsequent experiments were proliferated from the seeds.

In the first stage of our study, in order to test the effect of plant hormones on micropropagation, shoot tips of 1.0-1.2 cm in length were cut aseptically and cultured on basal MS medium supplemented separately with different concentrations of BAP and Kn (0.10, 0.15, 0.20, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 mg $l^{-1}$).

In the second stage, according to the results of our previous experiments, the media with BAP (0.5 mg $l^{-1}$) and Kn (1.5 mg $l^{-1}$) were seperately supplemented with various auxins (0.25 mg $l^{-}$ IAA, NAA, IBA) for shoot proliferation. All media were supplemented with 3% sucrose (w/v) and solidified with agar (0.55%, w/v, Agar-Agar (Sima). They were adjusted to the pH 5.8 prior to autoclaving (120°C for 20 min). The *in vitro* cultures were maintained at 25 ± 2°C for a 16 h photo period (40 µmol $m^{-2}$ $s^{-1}$) provided by mercury fluorescent lamps. All experiments were means of 16 replicates, and the experiments were repeated two times.

**Table 1.** The effects of concentrations of BAP on multiple shoot induction of *H. retusum* Aucher

| Concentrations of BAP (mg $l^{-1}$) | Avg No of shoots/ explant (Mean ±SE) | Avg length of shoots (cm) (Mean ±SE) |
|---|---|---|
| 0.00 | 1.12 ± 0.80 **f** | 3.58 ± 0.83 **a** |
| 0.10 | 8.18 ± 2.83 **e** | 2.74 ± 0.96 **cb** |
| 0.15 | 15.62 ± 7.75 **d** | 2.21 ± 0.66 **cd** |
| 0.20 | 18.87 ± 12.96 **d** | 1.96 ± 0.66 **d** |
| 0.25 | 38.93 ± 13.28 **b** | 2.71 ± 0.95 **cb** |
| 0.5 | 64.25 ± 15.75 **a** | 2.91 ± 0.48 **b** |
| 1.0 | 36.37 ± 7.69 **b** | 2.21 ± 0.43 **cd** |
| 1.5 | 34.18 ± 7.18 **b** | 2.98 ± 0.44 **b** |
| 2.0 | 33.93 ± 8.46 **b** | 2.98 ± 0.47 **b** |
| 2.5 | 26.25 ± 7.16 **c** | 2.83 ± 0.56 **b** |

Data recorded on the 6th week; 16 replicates/ treatmen; repeated twice. Values followed by the same letter are not significantly different (p = 0.05) according to student's *t*-test.

**Table 2.** The effects of concentrations of Kn on multiple shoot induction of *H. retusum* Aucher

| Concentrations of Kn (mg $l^{-1}$) | Avg No of shoots/ explant (Mean ±SE) | Avg length of shoots (cm) (Mean ±SE) |
|---|---|---|
| 0.00 | 1.12 ± 0.80 **d** | 3.58 ± 0.83 **d** |
| 0.10 | 11.25 ± 4.04 **c** | 7.30 ± 0.89 **a** |
| 0.15 | 11.37 ± 2.09 **c** | 6.80 ± 0.92 **b** |
| 0.20 | 11.81 ± 6.30 **bc** | 6.26 ± 0.86 **b** |
| 0.25 | 13.81 ± 5.29 **bc** | 6.49 ± 1.27 **b** |
| 0.5 | 15.68 ± 10.44 **bc** | 4.23 ± 0.87 **c** |
| 1.0 | 16.06 ± 6.80 **b** | 3.26 ± 1.49 **cde** |
| 1.5 | 27.87 ± 10.28 **a** | 3.65 ± 0.65 **cd** |
| 2.0 | 26.00 ± 14.26 **a** | 3.99 ± 1.22 **cd** |
| 2.5 | 35.68 ± 16.73 **a** | 3.05 ± 0.44 **e** |

Data recorded on the 6th week; 16 replicates/ treatment; repeated twice. Values followed by the same letter are not significantly different (p = 0.05) according to student's *t*-test.

## Results and discussion

### *The effects of different concentrations of BAP and Kn on* multiple shoot induction *of H. retusum Aucher*

The application of molecular approaches with medicinal plants would also benefit from the development of cell, tissue and organ culture systems for *in vitro* growth and regeneration of medicinal plants. In addition, such tissue culture systems could also prove

**Table 3.**The effects of combinations of BAP plus auxin on multiple shoot induction of *H. retusum* Aucher

| Treatment | Avg No of shoots/ explant (Mean ±SE) | Avg length of shoots (cm) (Mean ±SE) |
|---|---|---|
| 0.5 mg $l^{-1}$ BAP | 64.25 ± 15.75 **a** | 2.91 ± 0.48 **b** |
| 0.5 mg $l^{-1}$ BAP + 0.25 mg $l^{-1}$ NAA | 35.93 ± 13.87 **c** | 2.98 ± 0.47 **ab** |
| 0.5 mg $l^{-1}$ BAP + 0.25 mg $l^{-1}$ IAA | 42.68 ± 14.43 **bc** | 3.22 ± 0.54 **ab** |
| 0.5 mg $l^{-1}$ BAP + 0.25 mg $l^{-1}$ IBA | 54.12 ± 15.94 **ba** | 3.36 ± 0.57 **a** |

Data recorded on the 6th week; 16 replicates / treatment; repeated twice. Values followed by the same letter are not significantly different (p = 0.05) according to student's *t*-test.

**Table 4.** The effects of combinations of Kn plus auxin on multiple shoot induction of *H. retusum* Aucher

| Treatment | Avg No of shoots/ explant (Mean ±SE) | Avg length of shoots (cm) (Mean ±SE) |
|---|---|---|
| 1.5 mg $l^{-1}$ Kn | 27.87 ± 10.28 **a** | 3.65 ± 0.65 **a** |
| 1.5 mg $l^{-1}$ Kn + 0.25 mg $l^{-1}$ NAA | 11.31 ± 6.15 **b** | 2.58 ± 0.68 **b** |
| 1.5 mg $l^{-1}$ Kn + 0.25 mg $l^{-1}$ IAA | 29.31 ± 16.34 **a** | 2.71 ± 0.44 **b** |
| 1.5 mg $l^{-1}$ Kn + 0.25 mg $l^{-1}$ IBA | 32.37 ± 17.56 **a** | 3.68 ± 0.45 **a** |

Data recorded on the 6th week; 16 replicates / treatment; repeated twice. Values followed by the same letter are not significantly different (p = 0.05) according to student's *t*-test.

useful for large-scale biotechnological production of medicinal plant phytochemicals (Briksin, 2000). Furthermore, uniform plant growth with consistent plant material can be achieved, plants can be grown in sterile, standardized conditions and are free from biotic and abiotic contamination.

So far, no study has been reported on *in vitro* propagation, callus culture and secondary metabolites for this plant. This study describes the basic procedures for the establishment on multiple shoot induction of *H. Retusum* Aucher. Therefore, seeds were germinated (germinating rate about 90%) on the hormone-free medium within 7–10 days. Following germination, the seedlings (1.0–1.2 cm in stem length) were separately transferred to MS medium supplemented with 0.10-2.5 mg $l^{-1}$ of BAP and Kn (Figure 1A). The formation of new shoots was observed in all media studied except in the control group (hormon-free medium), indicating that *H. retusum* Aucher is highly responsive to plant growth regulators. Regeneration frequency, mean number and length of shoots per explant were recorded after all hormone experiments. In the first stage of our experiment, the number of shoots changed, depending on the different concentrations of BAP. When the number of shoot was compared, there were statistically significant differences among the concentrations of BAP tested (Table 1). The highest and the lowest number of shoots were obtained on the medium supplemented with 0.5 mg $l^{-1}$ (Figure 1B) and 0.10 mg $l^{-1}$ of BAP (64.25 - 8.18 shoot/explant, respectively). The high concentrations of BAP (2.0 or 2.5 mg $l^{-1}$) led to vitrification, callus formation and red colours in stems and leaves, although no change was observed in the appearance of shoots obtained from low concentrations of BAP (0.10- 0.5 mg $l^{-1}$).

All the investigated concentrations of Kn showed shoot production. However, the best result was obtained on the medium supplemented with 1.5 mg $l^{-1}$ Kn (Figure 1C). From the results presented in Table 2, it appears that the number of shoot rises by increasing the concentration of Kn. However, smaller and shorter shoots were formed as the concentration of Kn increased in the culture medium. Excessive shoot length and root formation were observed on the medium containing low concentrations of Kn (from 0.10 to 0.25 mg $l^{-1}$) (Figure 1D).

The results indicated that the highest shoot number formed on the medium supplemented with 2.5 mg $l^{-1}$ of Kn (35.68 shoots/explant) (Figure 1E). However, the morphological characteristics of shoots in this medium were not similar to plants growing in a natural environment and led to vitrification in shoots. Therefore, a culture medium with 1.5 mg $l^{-1}$of Kn was sufficient to produce multiple shoot and an average of 27.87 shoots formed from each explant in this medium within six weeks (Table 2). In the morphological observations, Kn concentrations surpassed other media (BAP concentrations) in terms of mean shoot length, leaf width and root formation.

In comparison of all treatments of two cytokinins with control group (Table 1-2), it was determined that the medium should be supplemented with exogenous hormones (PGRs) for new shoot formation.

In conclusion, shoots were successfully propagated after two subcultures in the presence of BAP or Kn. Among the cytokinins (BAP, Kn concentrations), BAP was reported to be more efficient than Kn in promoting shoot formation. Our findings are compatible with those of Cellárová et al. (1992), who reported that in *H. perforatum*, BAP was found to be the most efficient in shoot formation when excised parts of seedlings were used. Also, for *H. perforatum* L., BAP was found to be the most efficient in promoting shoot regeneration when leaves (Pretto and Santarem, 2000) were used as the explant.

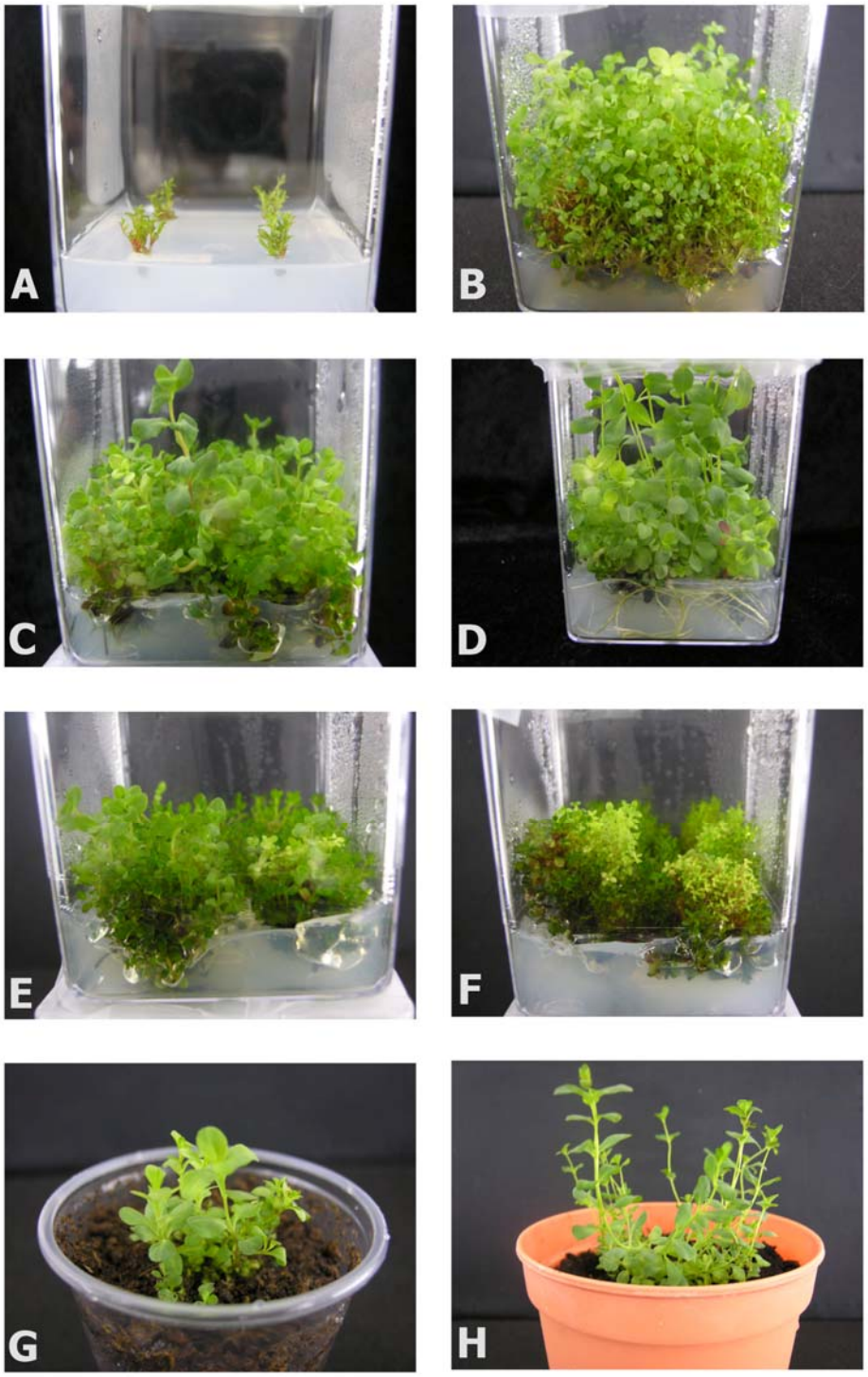


**Fig 1.** *In vitro* multiple shoot induction of *Hypericum retusum* Aucher.
**1A)** Aspect of shoots were germinated from mature seeds of ***H. retusum*** Aucher,
**1B)** Development of shoots on MS medium containing 0.5 mg $l^{-1}$ BAP.
**1C)** Development of shoots on MS medium supplemented with 1.5 mg $l^{-1}$ Kn.
**1D)** Development of shoots on MS medium containing 0.10 mg $l^{-1}$ Kn.
**1E)** Aspect of shoots on MS medium supplemented with 2.5 mg $l^{-1}$ Kn.
**1F)** Development of shoots on MS medium containing auxin+ cytokinin.
**1G)** Acclimatized plantlets 2 weeks after transfer to greenhouse.
**1H)** *In vitro* propagated plantlets 4 months after transfer to pots.

***The effects of combinations of cytokinins plus auxin on multiple shoot induction of H. retusum Aucher***

Shoots were cultured on MS medium supplemented with various combinations of cytokinins and auxins (0.5 mg $l^{-1}$ BAP + 0.25 mg $l^{-1}$ IAA, NAA and IBA, 1.5 mg $l^{-1}$ Kn + 0.25 mg $l^{-1}$ IAA, NAA and IBA). All the treatments with auxin produced shoots along the culture period. However, regardless of the auxin types used, intensive vitrification and yellowing of the explants were observed in all these treatments by the sixth week of culture (Figure 1F). In general, the use of auxins was not effective in our experiments for shoot formation.

According to the data presented in Table 3, similar results were obtained on medium containing BAP + NAA and BAP+IAA for shoot number. There were no statistical differences between the two groups regarding shoot number and shoot length ($p>0.05$).

As seen in Table 3, the best result in view of number and length of shoots obtained on MS medium supplemented with 0.5 mg $l^{-1}$ BAP + 0.25 mg $l^{-1}$ IBA (54.12 shoot/explant). However, the use of only BA on medium proved more beneficial than the combination of BAP and IBA for shoot formation. Results in Table 4 showed that there were no significant differences between tested groups (Kn + auxins) for shoot formation.

The effects of auxins and cytokinins on shoot multiplication have been reported earlier for *Hypericum* species (Cellárová et al. 1992; Moura, 1998). BA, alone or in combination with NAA, is known to be an effective shoot inducing agent in different species (Blakesley and Constantine, 1992). Santarém et al (2003) reported that explants cultivated on medium containing BA and NAA resulted in 40.6 shoots per explant after 60 d in culture. In contrast to our findings the best results were obtained on medium containing BAP+ IBA with a shoot number of 54.12 per explant for *H.retusum.* Although a higher shoot number was obtained from this medium, there were no significant differences compared to the control group (0.5 mg $l^{-1}$ BAP), and the use of BAP alone was the most efficient for shoot propagation. This is opposite to the observation of Moura, (1998) for *H. foliosum* and of Mederos, (1991) for *H. canariensis,* where the highest number of shoots was obtained on a media supplemented with BAP and NAA.

As seen in Table 3, The best result in view of number and length of shoot obtained on MS medium supplemented with 0.5 mg $l^{-1}$ BA + 0.25 mg $l^{-1}$ IBA (54.12 shoot/explant). However, the use of only BA on medium proved more beneficial than the combination of BAP and IBA for shoot formation.

***Acclimatization***

The rooted plantlets were transferred to non-aseptic conditions for acclimatization in pots with a moist mixture of sand and perlite (1:1). They were then transferred to soil in the growth room for a few days and later were transferred to a green house. The acclimatization potential was 80% (Figure 1G, H).

**Conclusion**

Tissue culture technology offers an alternative method for the conservation of germplasm as well as micropropagation of medicinally important plant resources. To the best of our knowledge, no report is available on *in vitro* propagation of *Hypericum retusum* Aucher. Therefore, this research describes a new system suitable for the multiple shoot induction of *H. retusum* Aucher.

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