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# MICROPROPAGATION OF *ANTHEMIS PESTALOZZAE* BOISS. FROM COTYLEDONARY LEAF AND LEAF EXPLANTS

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**Abstract**

*Anthemis pestalozzae* Boiss. is rare endemic plant of Southern Eastern Turkey. Biotechnological approaches are desired as alternative for *ex situ* conservation. This study aimed at evaluating factors affecting seed dormancy release and micropropagation of *A. pestolozzae*. The seed dormancy of *A. pestalozzae* was released with 2 mg/l $GA_3$ treatment under dark conditions. Cotyledonary leaf and leaf explants pretreated with 50 mg/l BAP for 72 hrs were maintained on MS medium supplemented with different combinations of BAP and NAA or without any hormone. The maximum number of (12.42) shoots were induced on leaf explants using MS medium containing 1 mg/l BAP + 0.5 mg/l NAA. Whereas, maximum number of shoots on cotyledonary leaf explants were 5.00 on MS medium containing 1.5 mg/l BAP + 0.75 mg/l NAA. Well developed shoots were rooted on half strength of MS medium containing 0.5 mg/l IBA. The rooted and acclimatized plants flowered under greenhouse conditions.

## Introduction

Many plant species among the genus *Anthemis* are characterized as rare, endemic or threatened (Tubives 2015). *Anthemis pestalozzae* Boiss. (Asteraceae) is annual endemic species found on the volcanic, limestone projections and hilly slopes spread over southern Anatolia. It was possible to see scattered large reserves of the plant a few years back; however, overexploitation of this endemic plant from natural reserves primarily for ornamental plant industry locally has left negative impacts on the populations of the plant (Arslan *et al*. 2002). A little attention could help in making use of this beautiful ornamental plant for low cost arid landscaping as it is resistant to hard climatic conditions.

The species of the *Anthemis* genus are widely used in the pharmaceutics, cosmetics and food industry. The flowers of the genus have well-documented use as antiseptic and healing herbs, the main components being natural flavonoids and essential oils (Vaverkova *et al.* 2007).

The economic importance is due to the production of *Anthemis* oil as digestive, gastrointestinal spasmolytic, anti-inflammatory, and sedative agent (Grace 2002). *Anthemis* essential oil is among versatile popular oils used in aromatherapy. In Europe extracts, tinctures, and teas are widely used as anti-inflammatory, antibacterial, antispasmodic, and sedative agents. Extracts are used to allay pain and irritation, clean wounds and ulcers, and aid prevention as well as therapy of irradiated skin injuries, treatment of cystitis and dental afflictions (Grace 2002). The antimicrobial activity of the essential oils and different extracts from several *Anthemis* species has been reported before (Holla *et al.* 2000, Grace 2002) Essential oils are used to get relief from pain and irritation, clean wounds and ulcers. The antimicrobial activity of the essential oils and extracts from several *Anthemis* species has also been reported (Uzel *et al.* 2004).

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Owing to its diversified uses the demand for anthemis oil is ever increasing. Decline of potential for the production of active principle or essential oil content is a common feature with cultivation of many aromatic plants including *Anthemis* species.

There have been three reports on the tissue culture of *Anthemis* species using various explants that mainly describe production metabolic, genetic transformation, germplasm conservation or plant micropropagation techniques (Echeverrigaray *et al.* 2000, Erdag and Emek 2005, Erdag and Emek 2009). There has been no report describing *in vitro* adventitious shoot regeneration of *A. pestalozzae.*

Development of biotechnological methods could serve as an alternative for *ex situ* multiplication and conservation of the endemic or threatened plants (Parmaksiz and Khawar 2006).

This study aimed at finding *in vitro* biotechnological approaches to break seed dormancy and establish an effective regeneration protocol for *A. pestalozzae* using preconditioned leaf and cotyledon leaf explants on MS medium containing combinations of BAP - NAA for rapid and large-scale propagation of uniform plants for field culture; which has never been reported earlier.

**Materials and Methods**

The seeds of *A. pestalozzae* were collected from the section plant taxonomy of the Department of Biology, Gazi University, Ankara, Turkey during 2012. The seeds have severe dormancy and poor germination.

The seeds were surface sterilized using 20% commercial bleach (5% NaOCl, Ace -Turkey) for 7 min in laminar flow hood followed by 5 × 3 min rinsing with sterilized double distilled water.

The sterilized seeds were divided into 5 groups. First set (control) was directly cultured on agar solidified MS medium (Murashige and Skoog 1962) for germination. The sterilized seeds of other 4 groups were primed with 0.5, 1, 1.5 and 2 mg/l (liquid) $GA_3$ in sterilized Falcon tubes in dark with horizontal shaking for 48 hrs at 24 ± 2ºC at 110 rpm. The seeds that protruded 0.2 mm radicle were counted as germinated. After optimizing best concentration of $GA_3$, this concentration was used in rest of the experiments to obtain germinated seedlings for explants for regeneration studies. Both explants were pre-conditioned with 50 mg/l BAP and incubated for 72 hrs in dark at 24$^{o}$C.

The BAP pre-conditioned explants were post conditioned on MS medium (control) or MS medium containing 0.5, 1, 1.5, 2 mg/l BAP + 0.5, 0.75, 1 mg/l NAA; (13 combinations, Table 1) for two weeks followed by culture for two weeks on MS medium to achieve micropropagation. Well developed shoots were rooted on half strength of MS medium contaınıng 0.5 mg/l IBA.

The pH of all cultures was adjusted to 5.7 - 5.8 before adding 0.7% (w/v) agar (Sigma type A), autoclaving at 121°C temperature and 104 KPa pressure for 20 min. All cultures were grown at 24 ± 2°C under cool white fluorescent light providing (35 µMol photons $m^{-2}s^{1}$) light intensity and 16 hrs light photoperiod unless mentioned otherwise.

These plants were planted in peat moss and maintained in the Sanyo versatile environmental test chamber at 24 ± 2°C under 16 hrs light (3000 lux light) photoperiod. They were given 50 ml water every 2 days to maintain proper soil humidity and plant turgor.

Each treatment contained 60 explants for both seed germination and explant regeneration. Each treatment was divided into 15 replicates containing 4 explants each (4 explants × 15 replicates = 60 explants). The data was subjected to GLM univariate analysis using SPSS 12.0 for windows statistical software and the *post hoc* tests were performed using DMRT.

**Results and Discussion**

No seed germination was noted on MS medium (Control). Seed germination of 1.00, 30.67, 70.77 and 100.00% was noted on 0.5, 1.0, 1.5 and 2 mg/l $GA_3$ priming, respectively (Fig. 1) after one week. Seed priming of 2 mg/l $GA_3$ (highest germination) was used in all subsequent experiments to grow seedlings after culture on MS medium and obtain cotyledon and leaf explants. The results showed that the seeds of *A. pestalozzae* had high non deep physiological seed dormancy that could not be germinated on MS medium. This was further confirmed by observing a gradual increase in germination percentage of treated seeds from 0.5 to 2 mg/ $GA_3$ (4 concentrations). One hundred per cent seed germination was noted on 2 mg/l $GA_3$ priming in agreement with Baskin and Baskin (2004) and Sarihan *et al.* (2005); who found positive effects of $GA_3$ treatments for early germination of *Osmorhiza claytonii* and *Plantago lanceolata* seeds, respectively.

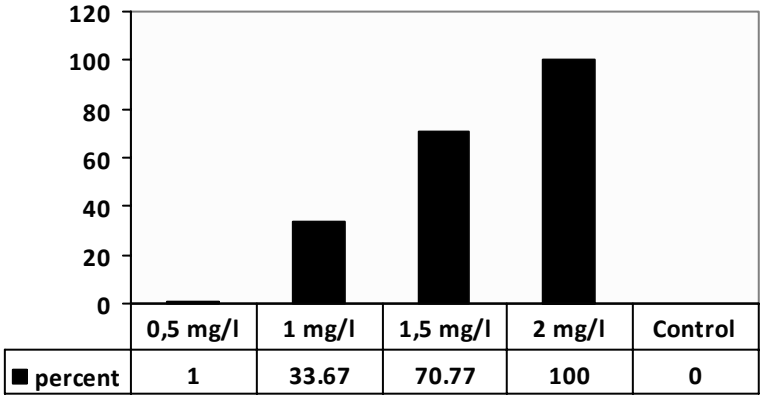


Fig. 1. Effect of seed priming with variable concentrations of $GA_3$ on seed germination percentage of *A. pestalozzae*.

It is well documented that BAP used singly or in combination with NAA are very effective in increasing *in vitro* shoot regeneration and growth (Khawar *et al.* 2005, Ozel *et al.* 2008). The leaf and cotyledon leaf explants taken from one week old young seedlings induced excellent shoot regeneration. Leaf explants were better compared to cotyledon leaf explants in terms of shoot regeneration percentage; where shoot regeneration percentage ranged 66.67 - 100 (Table 1). Minimum shoot regeneration percentage was noted on BAP preconditioned leaf explants on post conditioning with MS medium containing 1 mg/l BAP + 1 mg/l NAA. Shoot regeneration percentage remained 100 on control treatment and 8 out 12 different concentrations of BAP+NAA for leaf explant.

Shoot regeneration percentage on cotyledon leaf explant remained 33.33 on control. Shoot regeneration percentage after post conditioning with variants of BAP + NAA after BAP preconditioning of cotyledon leaf explants ranged 33.33 to 91.67. Maximum regeneration was noted on explants post conditioned on MS medium containing 1.5 mg/l BAP + 0.75 mg/l NAA. Minimum regeneration of 33.33% was noted on explants post conditioned on MS medium containing 1 mg/l BAP + 0.5 mg/l NAA and MS medium (control).

Preconditioned leaf explants induced higher number of shoots per explant compared to number of shoots induced on cotyledon leaf explants. Number of shoots per preconditioned leaf

explant ranged 2.92 - 12.42 (Table 1). Maximum number of 12.42 shoots per BAP preconditioned leaf explant was recorded on post conditioning with MS medium containing 1 mg/l BAP + 0.5 mg/l NAA (Fig. 2a). Minimum number of 2.92 shoots per BAP preconditioned leaf explant was recorded on post conditioning with MS medium containing 1 mg/l BAP + 1 mg/l NAA. These shoots were less in number when compared to number of shoots per explant (6.50) induced on BAP preconditioned leaf explants cultured on MS medium (control).

Contrarily number of shoots per BAP preconditioned cotyledon leaf explant ranged 0.41 to 5.50. Maximum number of shoots per BAP preconditioned cotyledon leaf explant was noted on post conditioning with MS medium containing 1.5 mg/l BAP + 1 mg/l NAA (Fig. 2b). Minimum number of shoots on BAP preconditioned cotyledon leaf explant was noted on post conditioning with MS medium containing 1 mg/l BAP + 0.5 mg/l NAA and MS medium (control). Within each of 4 groups of BAP + NAA concentrations; any concentration of BAP + 1 mg/l NAA had the most positive increase in shoots length.

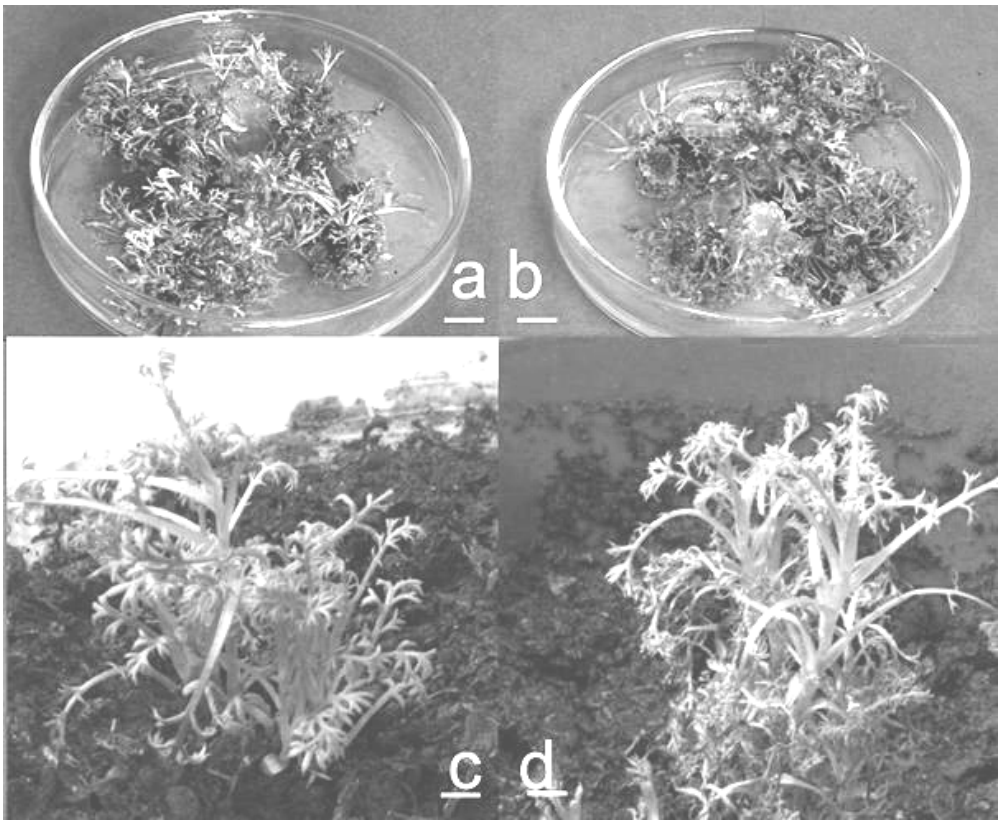


Fig. 2. Micropropagation of *A. pestalozzae:* (a) Maximum number of 12.42 shoots per 50 mg/l BAP preconditioned leaf explant recorded on MS medium containing 1 mg/l BAP + 0.5 mg/l NAA, (b) maximum number of 5.50 shoots per 50 mg/l BAP conditioned cotyledon leaf explant was noted on MS medium containing 1.5 mg/l BAP + 0.75 mg/l NAA, (c, d) the rooted plants acclimatized in the Sanyo versatile environmental test chamber at 24 ± 2°C under 3000 lux light and 16 hrs light photoperiod.

**Table 1. Effects of various concentrations of BAP-NAA on shoot regeneration and number of shoots per explant of *A. pestalozzae*.**

| Treatments | | Shoot regeneration percentage | | Number of shoots per explant | | Shoot length (cm) | |
|---|---|---|---|---|---|---|---|
| BAP (mg/l) | NAA (mg/l) | Leaf explant | Cotyledon leaf explant | Leaf explant | Cotyledon leaf explant | Leaf explant | Cotyledon leaf explant |
| 0.50 | 0.50 | 100.00 a[1]A[2] | 50.00 bcB | 11.17 abA | 1.08 bcdB | 1.47 bB | 2.48 abA |
| 0.50 | 0.75 | 100.00 aA | 58.33 bcB | 9.25 abcA | 0.83 cdB | 1.37 bA | 1.55 cdA |
| 0.50 | 1.00 | 100.00aA | 50.00 bcB | 7.00 cdeA | 3.75 abcdB | 1.16 bB | 3.02 aA |
| 1.00 | 0.50 | 100.00 aA | 33.33 cB | 12.42 aA | 0.41 dB | 1.77 bA | 0.83 eB |
| 1.00 | 0.75 | 100.00 aA | 58.33 bcB | 8.25 bcdA | 2.25 abcdB | 1.48 bA | 1.26 cdeA |
| 1.00 | 1.00 | 66.67 bA | 41.67 cB | 2.92 fA | 4.50 abcB | 1.27 bA | 1.20 cdeA |
| 1.50 | 0.50 | 91.67 abA | 50.00 bcB | 5.45cdefA | 1.50 bcdB | 1.78 bA | 1.88 bcA |
| 1.50 | 0.75 | 91.67 abA | 91.67 aA | 3.92efB | 4.42abcB | 1.69 bA | 1.66 cdA |
| 1.50 | 1.00 | 100.00 aA | 75.00abB | 4.58 defA | 5.50 aA | 1.69 bA | 1.79 bcA |
| 2.00 | 0.50 | 100.00 aA | 50.00 bcB | 11.92 abA | 2.58 abcdB | 1.56 bA | 1.05 deB |
| 2.00 | 0.75 | 91.67 abA | 75.00 abB | 9.17 abcA | 3.41 abcdA | 1.26 bB | 1.76 cA |
| 2.00 | 1.00 | 100.00 aA | 58.33bcB | 3.92efA | 4.92 abB | 1.26 bA | 1.32 cdeA |
| Control (1×MS medium) | | 100.00 aA | 33.33cB | 6.5cdefA | 0.75 cdB | 3.37 aA | 0.80 eB |

[1]Means of all values shown by different letters in a same column are statistically different using Duncan's multiple range test at 0.05 level of significance. [2]Means of all values shown by different capital letters in a same row are statistically different using t test at 0.05 level of significance.

The shoots that were regenerated on BAP preconditioned cotyledon leaf explants on any combination of BAP + NAA were comparatively longer compared to shoots regenerated on BAP preconditioned leaf explants. BAP preconditioned leaf explants induced shoot length of 3.37 cm on MS medium (control). Shoot length on rest of BAP preconditioned leaf explants ranged 1.26 - 1.78 cm (control – Table 1). The longest shoots (1.78 cm) on BAP preconditioned leaf explant were recorded on MS medium containing 1.5 mg/l BAP + 0.5 mg/l NAA. BAP preconditioned cotyledon leaf explants induced shoot length of 0.80 cm on MS medium (control). Shoot length on rest of BAP preconditioned cotyledon leaf explants ranged 0.83 - 3.02 cm (Table 2). The longest shoots on BAP preconditioned cotyledon leaf explant were recorded on MS medium containing 0.5 mg/l BAP + 1 mg/l NAA. Tissue culture experiment results were very promising as well. Leaf explants was better compared to cotyledon leaf explants in terms of frequency of shoot regeneration. One hundred per cent shoot regeneration was noted on BAP conditioned leaf explants containing 8 out 12 concentrations BAP + NAA or MS medium used singly. Maximum number of 12.42 shoots on BAP preconditioned leaf explant was noted on post conditioning with MS medium containing 1 mg/l BAP + 0.5 mg/l NAA. Even BAP conditioned leaf and cotyledon leaf explants regenerated variable number of shoots per explant on MS medium. The shoots that were regenerated on BAP conditioned cotyledon leaf explants on any combination of BAP + NAA were comparatively longer compared to shoots regenerated on BAP conditioned leaf explants. Contrarily, Erdag and Emek (2009) had maximum number of 6.70 ± 1.05 adventitious shoots/explant on leaf explants of *Anthemis xylopoda* using 0.5 mg/l BAP.

*Anthemis pestalozzae* rooted readily in MS medium containing half strength of MS medium with 0.5 mg/l IBA. Previous studies emphasize that an increase in root length and number is very

important for acclimization under *ex vitro* conditions. Both number of roots and their length help in easy uptake of water, and nutrients under *in vitro* conditions (Barpete *et al.* 2014). The results of this study showed higher number of roots on shoots regenerated on leaf explants compared to rooting of shoots regenerated on cotyledon explant using both 0.5 mg/l IBA in half strength of MS medium. The results of the study showed significant effect of type of explant on root induction. The maximum number (4) of roots were observed on leaf induced shoots followed by 2.34 roots on cotyledon leaf induced shoots using half strength of MS medium.

The germinated seedlings had no problem in acclimatization and grew with profuse flowering in the greenhouse. The results of this study showed that BAP conditioning for 72 hrs was enough for *in vitro* shoot regeneration. The results of this study are in agreement with previous studies by Aasim *et al.* (2009) in cowpea. They showed that BAP conditioning is very helpful in shoot induction in cowpea.

The results of this study revealed role of $GA_3$ priming in breaking seed dormancy of *A. pestalozzae.* It can be concluded that pre and post conditioning treatments can play a crucial role in regulating shoot and root development in *A. pestalozzae* under *in vitro* conditions. The reported protocol established a new vista in *A. pestalozzae* biotechnology and will definitely serve as the foundation in possible mass production of this important plant that will help in widening the scope of this plant in arid landscaping with persistent water deficit.

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