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Efficient Embryogenic Callus From Filaments With Anther In *Thermopsis turcica*

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Abstract

Thermopsis turcica is an endemic endangered species. The establishment of an improved micropropagation method is very important to preserve this species *in vitro* conditions. For this reason, the effects of different media and temperature pretreatments on the stamens of *T. turcica* planted at Nezahat Gokyigit Botanical Garden in Istanbul were investigated. *T. turcica* filaments with anther were excised and cultivated *in vitro* with Murashige and Skoog's basal media supplemented with Naphthaleneacetic acid (4 mg L^{-1}), Benzylaminopurine (0.1 and 0.5 mg L^{-1}), and $AgNO_3$ (15 mg L^{-1}) at 4, 28 and 37°C for a week under complete darkness. Moreover, to determine the effects of activated charcoal on regeneration, a medium containing 0.25% activated charcoal was also used. A week after culture initiation, all samples were then incubated at 28°C for a month under a 16 h photoperiod. The most promising callus formation was observed in filaments cultured on the media with 0.1 mg L^{-1} Benzylaminopurine (100%) pretreated at 37°C a week after culture initiation; however, the development of embryos from callus could also be visualized from the filaments cultured on the medium with 0.1 mg L^{-1} Benzylaminopurine (50%) pretreated at 4°C. None of the activated charcoal treatments yielded positive results regarding callus formation. Instead they negatively affected callus formation in the mixture media. Furthermore, control groups that were incubated on MS medium free from growth regulators did not show any growth. According to a literature review on *T. turcica*, no stamen culture has been performed to date.

Keywords: Callus, embryo, filament, *Thermopsis turcica*

Introduction

Thermopsis turcica is the member of the subfamily Papilioideae of Fabaceae. This species is a critically endangered endemic plant species in Turkey. Furthermore, it is quite unique as it has a gynoecium of 2-4 functional carpels, which does not occur in other members of the subfamily Papilioideae. This species has 10 free stamens (Tan et al. 1983; Davis et al. 1988; Erik and Tarikahya, 2004; Vural, 2009; Ekim et al. 2000; Ozdemir et al. 2008; Tekdal and Cetiner 2014). Despite the importance of *T. turcica* by means of its valuable females for crop breeding purposes and being in danger of extinction, a few studies have been conducted on the *in vitro* propagation of this species.

Stamen culture is also one useful technique to propagate this species *in vitro* conditions. The culture of filaments with anther is known as stamen culture. Stress conditions such as heat, light and the developmental stage of flower buds are very important components of stamen culture (Smykal, 2000; Stewart, 2008; Ferrie and Caswell, 2014).

Based on our literature review, no research on *T. turcica* to date has been conducted on stamen culture in *T. turcica*, however, research on its propagation is also very important. Because of this, stamen culture was conducted as an alternative technique for its propagation.

The objective of this research is not only provide information on anther culture but also on filament culture. Moreover, the purpose of the present study is to analyze which parameters, such as flower bud size, temperature and medium content, are high effective on stamen culture of *T. turcica* and which one is more beneficial for culture.

Materials and Methods

Plant Material

In this study, flower buds at various ages were used as explant sources. To identify the

development stages of pollen, microspores were isolated from the buds and then dyed with acetocarmine. Photographs of these were taken under a fluorescent microscope (Figure 1; A, B, C). Selected flower bud size and anthers are shown in Figure 1; A1, B1, C1.

The flower buds of *T. turcica* were harvested at Nezahat Gokyigit Botanical Garden in Istanbul in 2014. *Thermopsis* flower buds were sterilized; stamens were then isolated and cultured.

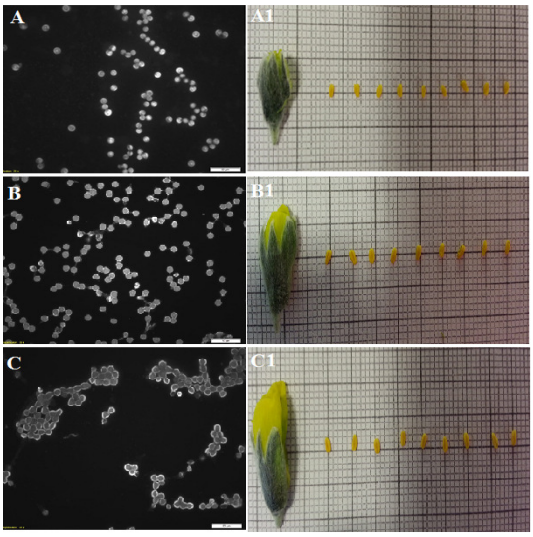

Figure 1. A, B and C show microsopores of *T. turcica* (scale bar is 50 µm); A1, B1 and C1 show anthers and flower buds at various ages of *T. turcica*

Media and Culture Conditions

Ten filaments with anther were placed into 100x15mm petri dishes containing basal MS basal medium with MS vitamins, BA (0.1 and 0.5 mg L^{-1}), NAA (4 mg L^{-1}), $AgNO_3$ (15 mg L^{-1}), 3% (w/v) sucrose and 0.75% (w/v) agar. In addition to this medium, to understand the effect of activated charcoal, a medium including 0.25% (w/v) activated charcoal was also used. Control cultures were incubated on a hormone free Murashige and Skoog's (MS) basal medium. Cultures were incubated at 4, 28 and 37°C at dark for a week. After a week, all samples were incubated in a growth room at 28°C for a month. *In vitro* grown embryogenic callus were transferred into a MS medium, free from plant growth regulators, for organogenesis. These embryogenic calli were then incubated at 28°C under 16 h photoperiod provided by cool white fluorescent lamps.

Methods

Surface Sterilization

The flower buds were dipped into 70% ethanol for three minutes and kept in 10% sodium hypochlorite for 10 minutes. The flower buds were then rinsed three consecutive times with sterile distilled water and blotted dry in a laminar flow cabinet.

***In vitro* Tissue Culture**

After surface sterilization, filaments with anther were extracted from the buds under a stereomicroscope and placed then on the medium. The petri dishes containing the stamens were cultured for approximately one month in a growth chamber. The experiment was set for a total of six treatments; each treatment was carried out in triplicates containing ten explants in each culture medium. The regeneration ability of each treatment was then scored weekly for a period of four weeks. The data on callus creation and embryogenic callus formation was collected.

Data Analysis

To determine the mean of callus regeneration and embryo formation, Minitab 17 program was used.

Results and Discussion

In our study, we used heat treatment (4, 28 and 37°C) and two different media consisting of varying BA concentrations (0.1 and 0.5 mg L^{-1}), NAA (4 mg L^{-1}) and $AgNO_3$ (15 mg L^{-1}), as stress stimuli, and we used different bud size (Figure 1; A1, B1 and C1) as culture initiation.

After a week in the culture, in all samples except for control groups cultured on MS medium free from growth regulators, callus creation was observed. On the other hand, when activated charcoal was added to the medium, no callus formed.

According to our findings, a comparison of the stamen growth stage showed that the stamens isolated from the flower buds shown in Figure 1; B1 has better callus induction and embryo formation from callus than did the stamens isolated from the flower buds shown in Figure 1; A1 and C1. The maxiumum callus regeneration was achieved in stamens isolated from the flower buds shown in Figure 1; B1 cultured on MS medium supplemented with 0.1 mg L^{-1} BA at 37°C (Figure 2).

The best embryogenic callus formation was observed from calli growing on MS media containing 0.1 mg L^{-1} BA at 4°C (Figure 3 and 4). The success of the experiment depended on the developmental stage of the stamens as well as temperature pretreatments.

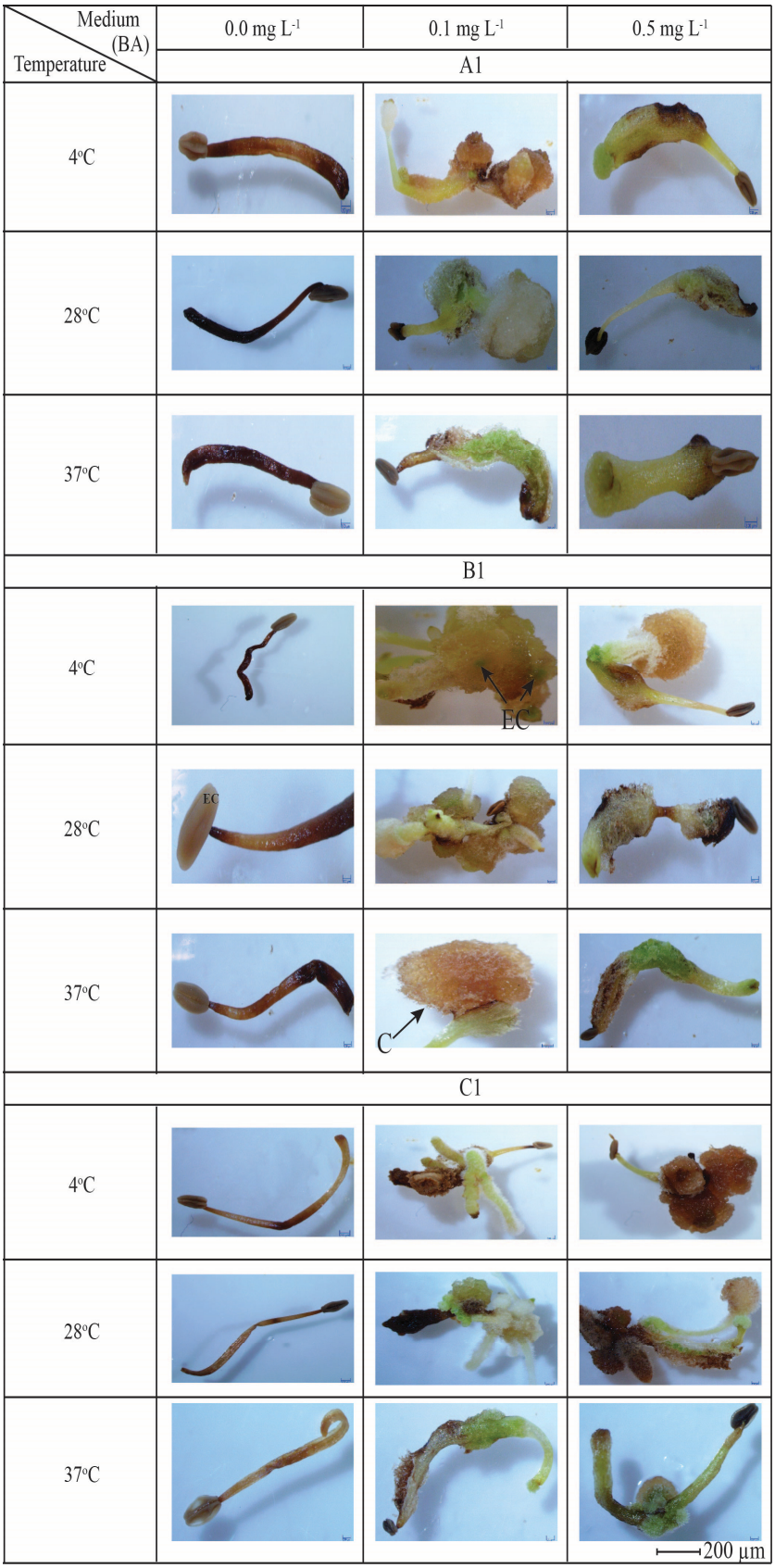

Figure 2. Stamens at different stages of their development (A1, B1, C1), different temperatures (4, 28 and 37°C) and BA concentrations (0.0, 0.1 and 0.5 mg L^{-1}) (arrows in B1; EC: Embryogenic callus, C: Callus)

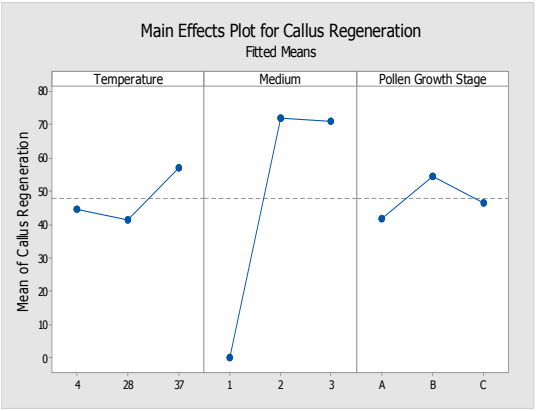

Figure 3. The mean of callus regeneration (Temperature: 4, 28 and 37°C; Medium: 1: Control, 2: MS with 0.1 mg L^{-1} BA, 3: 0.5 mg L^{-1} BA; Pollen Growth Stage: A, B and C shown in Figure 1)

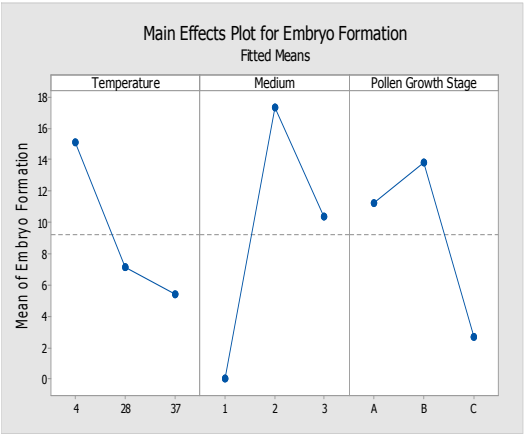

Figure 4. The mean of embryo formation on callus (Temperature: 4, 28 and 37°C; Medium: 1: Control, 2: MS with 0.1 mg L^{-1} BA, 3: 0.5 mg L^{-1} BA; Pollen Growth Stage: A, B and C shown in Figure 1)

According to this result, we identified which buds at the useful development stage could be selected for obtaining embryogenic callus in stamen culture. In earlier studies on microspore culture, microspore embryogenesis were obtained at high frequency in cold temperature treatment (Ferrie and Keller, 1995; Kasha et al. 2003; Ochatt et al. 2009).

According to our observations and the regeneration possibilites of the samples, there were significant differences in the callus regeneration and embryo formation between control groups that were cultured on MS medium free from hormone and all other samples that were cultured on MS medium added BA (0.1 and 0.5 mg L^{-1}) without activated charcoal. In all experiments, we could not obtain callus from anther. Any stress conditions, such as heat treatment, media formulation, culture condition and bud developmental stage, could have negative effects on the obtainment of microcalli from anther. The low concentration of BA (0.1 mg L^{-1}) in combination with the temperature treatment stimulated callus growth on filaments of *T. turcica.* On the contrary, in all samples cultured on the medium containing activated charcoal, no growth was observed in either anther or filament. Furthermore, in all experiments including control groups, callus from anther was not obtained and anthers turned into dark brown in colouration. In an earlier study on stamen culture, callus formation obtained from the filament of *Anemone coronaria* was cultured on the medium containing 0.1 mg L^{-1} BA (Ari, 2006). This result is similar to our result.

After two weeks on the medium, callus developed into embryo. As these embryos continued to grow, they did not develop into plantlets. For this reason, embryogenic calli were transferred into the MS medium free from growth regulators, and after a month in culture, no development could be observed.

In our study, in all tested media we used $AgNO_3$, and in all control groups cultured on the MS medium free from BA but including $AgNO_3$, callus formation was not observed. Thus, we can clearly indicate that $AgNO_3$ has not effected the stimulation of callus formation. On the other hand, BA highly stimulates callus induction.

As a result, we observed that temperature is an important factor in stamen culture. Heat treatment is the most common pretreatment used in microspore culture (e.g. *Brassica napus*: Pechan et al. (1991); *Brassica rapa* L. *ssp. chinensis:* Cao et al. (1994); *Capsicum annuum* L.: Kim et al. 2008)).

Some tissue culture may be time-consuming and requires a specific process prior to its culturing. In our study, we obtained callus in a week. As a consequence of the present study, it is seen that stamen culture may enable micropropagation of *T. turcica* in a relatively short period as compared to other tissue culture techniques. This is due to our ability to obtain callus from filament in a week.

Conclusion

Although there are several studies on *in vitro* propagation of *T. turcica*, there is currently no microspore, anther and stamen culture protocol for this species. From our findings on the stamen culture of *T. turcica*, it is shown that diploid, somatic callus and embryos can be obtained easily in a short period of time. On the other hand, we could not develop embryos obtained from calli into plantles. For this reason, further studies are

needed to analyze other embryo culture conditions.

Outcomes obtained during stamen culture in the present study will be a source for further studies on embryogenesis and anther culture which will subsequently develop new variaties of *T. turcica* for breeding programs.

Acknowledgements

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