The Journal of Animal & Plant Sciences, 24(6): 2014, Page: 1673-1679
ISSN: 1018-7081

IN-OVULE EMBRYO CULTURE OF *Thermopsis turcica*

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ABSTRACT

The possibility of micropropagation for the endangered *Thermopsis turcica* was investigated through an fruit ovule-embryo culture. This technique prevents embryo degradation at the early development stage, shortens breeding time, and enables efficient propagation of many plants from a single embryo, thus avoiding for breaking seed dormancy. Although a number of efficient propagation protocols of *T. turcica* has been reported for multiple shoot induction and plantlet regeneration from the seeds of *T. turcica*, to date no publication exists on any protocol on embryo culture of *T. turcica*. This paper reports on the optimisation of protocols for culturing of *T. turcica* fruit embryos. The protocol for embryo culture used in this research consisted of cracking the fruit, pitting it to remove the seed, excising the ovule-embryo from the seed, placing it on the medium modified from established Fabaceae embryo tissue culture media, extracting embryos from ovular integuments, and culturing. In the present study, extracted ovule-embryos were cultured on a Murashige and Skoog's medium supplemented with Zeatin (0.5 mg L^{-1}), Giberellic acid (0.5 mg L^{-1}), and Indole-3-acetic acid (0.2 mg L^{-1}). Excised embryos from the ovular integuments were maintained on Giberellic acid-free MS medium. During culturing, cotyledons began to arise and grow plantlets. Regenerated plantles were rooted on a MS medium with Indole-3-butyric acid (2.0 mg L^{-1}). These results show that embryo culture technique can be an alternative ex-situ conservation technique for *in vitro* propagation of the threatened rare plant species *T. turcica*.

Key words Micropropagation, fabaceae, seed, rare plant species, tissue culture media

INTRODUCTION

Thermopsis turcica Kit Tan, Vural & Kucukoduk (Fabaceae) is the critically endangered and endemic Turkish species located between the south-western part of Aksehir Lake and the southern part of Eber Lake (Tan *et al*. 1983; Davis *et al*. 1988; Cenkci *et al*. 2008). *T. turcica* is a hermaphrodite with golden yellow flowers, a long rhizome, and a 2-3 seeded legume fruit (Ozdemir *et al*. 2008) (Figure 1).

The uniform occurence of at least two free carpels of *T. turcica* is the first record in the subfamily *Papilioideae* (=*Faboideae*) of *Leguminosae* (Ozdemir *et al*. 2008; Cenkci *et al*. 2007, 2008, 2009).

However, almost all *T. turcica* populations are under heavy destruction due to the excessive agricultural use of lake water, the plant's habitat. Because of these factors, this endemic plant has been cited in the Red Data Book of Turkish Plants (Ekim *et al*. 2000) as critically endangered; the collection of *T. turcica* from its natural habitats has thus been banned in Turkey.

The plant's seed predator and risk of extinction (Cenkci *et al*. 2008, 2009; Vural 2009) thus necessitate the embryo culturation of this plant as an important alternative to other plant tissue culture techniques for germplasm conservation.

Due to the appearance of a 2-3 free carpellate-ovary, *T. turcica* possesses a valuable character in the breeding of fruit crops among the plant species of the family Fabaceae. This genotype is valuable as females. Unfavourable climatic and edaphic conditions as well as seed predation, however, prevent viable germinable seeds from *T. turcica*.

Literature on *T. turcica* indicates that to date no study exists regarding the embryo's culture of *T. turcica*. Therefore, this study proposes inducing *in vitro* germination of *Thermopsis* embryos extracted from various *T. turcica* parental lines so as to establish the successful culture of *Thermopsis* embryos in an artificial medium.

MATERIALS AND METHODS

SOURCE OF MATERIAL

Plant Material: *Thermopsis turcica* (Eber population) fruit seeds were collected from Istanbul's Nezahat Gokyigit Botanical Garden, in late June of 2011. Fruit seeds were sterilized, embryos were then isolated and cultured.

Plant Tissue Culture Media: Selections were made from media established Fabaceae embryo culture protocols by different research groups. A modified Cohen *et al*. (1984) medium which is a more complex for embryo development, was chosen for the ovule-embryo culture while for development of the *T. turcica* embryos into plantles, a Cenkci *et al*. (2008) modified medium was employed.

Fruit Sterilization: Fruits were dipped into 70% ethanol and kept in 20% sodium hypochlorite for 5 minutes. The fruits were then rinsed three times with sterile water in a laminar flow cabinet. After straining the water, seeds were pitted under sterile conditions. The ovule-embryos were isolated from the seeds and placed directly on the culture medium.

Ovule-Embryo Culture: Only one ovule-embryo was placed in a petri dish containing a modified Cohen *et al*. (1984) medium (see Table 1 for compositions), to avoid contamination by other samples. Only the micropyler end of the ovule was in direct contact with the medium. Control cultures were maintained on a hormone free Murashige and Skoog's (MS) basal medium. Ovule-embryos were cultured for 2 weeks in a growth chamber at 25 ± 1 °C under complete darkness.

Extraction of Embryos from The Ovul: Open ovules at the end opposite to that of the micropyle were cut and embryos from the ovular integuments were carefully excised. All embryos which were longer than 10 mm were cultured in an upright position with the medium that differed from that given in Table 1. The medium used for extracted embryos was free from Giberellic Acid (GA_3) and contained 0.5 mg L^{-1} Zeatin (ZEA) and 30 mg L^{-1} sucrose, reaching up to the hypocotyl for 2 weeks in a growth chamber at 25 ± 1 °C exposed under a 16 h photoperiod. GA_3 was not used for this step. After 2 weeks, the extracted embryos were transfered to test tubes containing a modified Cenkci *et al*. (2008) medium.

Regeneration of Plantlets from Extracted Embryos: The test tubes containing the embryos were cultured for approximately one month in a growth chamber so as to develop into plantlets; for this step, a modified Cenkci *et al*. (2008) medium composed of 4.3 g MS basal salt, 1 mg L^{-1} MS vitamin solution, 2.0 mg L^{-1} Indole-3-butyric acid (IBA), 30 g L^{-1} sucrose, and 7 g agar, was used.

The medium's pH was adjusted to 5.7 ± 0.2 before the addition of the gelling agent and autoclaved at 121°C for 15 min. As a control group, some samples were maintained on a MS medium without a plant growth regulator.

All samples were cultured in a growth chamber at 25 ± 1 °C exposed under a 16 h photoperiod (Table 2).

Rooting and Acclimatization: The excised shoots (~5 cm) from 4-week-old shoots were transferred to MS basal medium containing 2.0 mg L^{-1} (IBA) as a rooting medium. All samples were cultured as mentioned above for three weeks and transferred into plastic pots including sterile soil, after following the acclimatization method of Ripley and Preece (1986).

RESULTS AND DISCUSSION

Fruit sterilization: After surface sterilization described in Materials and Methods, seeds were removed (Figure 2 (A)) and then germinated. On the 7th day of culturing of embryos, their contamination cases were examined and, based on the sterilization method used in the study, no contamination was observed. There are some studies concerning the effects of treatments on germination of *Thermopsis* seeds (Norman 1996; Dreesen and Harrington 1997; Cenkci *et al*. 2007). In these studies, mechanical scarification and acid treatment implementation were applied on *Thermopsis* seeds for germination. To date only one group has applied acid scarification which stated that a 120 minutes sulfiric acid scarification is sufficient to obtain 98% seed germination (Cenkci *et al*. 2007). In the present sterilization, primarily sodium hypochloride was used as a sterilant on fruits; and the method proposed here was found to be effective for embryo culture because of the absence of a negative effect on embryo culture. In addition, during this sterilization stage, surface-sterilization was applied on fruits and thus, there was no seed damage from chemical use.

Ovule-Embryo Culture and Extraction of Embryos from The Ovule: After two weeks in the culture under complete darkness, all ovules planted in control groups turned brown and the embryos died. On the other hand, the embryos placed on the modified Cohen *et al*. (1984) remained viable. After the embryos were released, they were placed on MS medium free from GA_3 and containing 0.5 mg L^{-1} ZEA, 0.2 mg L^{-1} IAA and 30 mg L^{-1} sucrose. Cohen *et al*. (1984) recommend that zeatin should be added into the medium to rapidly grow embryos. In that study, the developmental stage of extracted embryos from ovules was a good variable, from globular to cotyledonary. By applying GA_3 into the ovule, medium sized viable embryos were obtained. In addition, we noticed in our study that all the legumes obtained from the Eber population contained healthy fruit seeds (Figure 2 (B)).

The main difference between the medium used in our study and that used by Cohen *et al*. (1984) is the concentration of zeatin. At the stage of ovule-embryo culture, the media were supplemented with 7.5 g L^{-1} agar in our study, whereas in the recommended protocol for Cohen *et al*. (1984), the media were supplemented with 9 g L^{-1} agar. In addition, the medium used for culturing excised embryos in our study, was supplemented with 0.5 mg L^{-1} ZEA, whereas in the Cohen *et al*. (1984) study, the media were supplemented wtih 0.2 mg L^{-1} ZEA. After cultured for two weeks on a modified Cohen *et al*. (1984) medium, the embryos developed rapidly (Figure 2 (C) and (D)). In contrast, we observed that the presence of plant hormones developed abnormally with the induction

of multiple buds without any rootlet (Figure 3 (A)). On the given medium, the shoots were elongated. Possibly, the root formation of regenerated *Thermopsis* shoots is affected by other unknown factors based on medium composition. On the other hand, no callus induction was observed. Mikulik (1999) explained that if only *in vitro* plant tissues lack callus proliferation, then, unaltered genetically stable plants can be regenerated. We observed that the plantlets are morphologically similar to the parents without callus formation. In addition, according to the previous research outcomes of the abundant studies on seed germination protocol, the seed's hard coat of *T. turcica* has caused strong physical dormancy (Ozdemir *et al*. 2008; Cenkci *et al*. 2009). As a result of their study on *T. turcica*, Cenkci *et al*. (2007) suggest a propagation strategy for *T. turcica* entailing removal of the hard seed coat so as to enable early seedling development.

On the basis of these results, the extraction of *Thermopsis* embryos could be enabled by the improvement of an *in vitro* technique of culturing embryos (Table 3). However, the research in this paper observes that an earlier germination and plantlet development because of the absence of time consuming actions of breaking seed dormancy and removing the seed's hard coat.

Regeneration of Plantlets from Extracted Embryos: In this study, the development of plantles from embryos was successful. Although shoot development was observed in plantlet development from embryos, no root induction occured when the Cohen *et al*. (1984) medium was used for subculturing in our experiments (Figure 3 (B)).

Due to our inability to observe root formation, a modified Cenkci *et al*. (2008) medium was used to obtain plantlet with rootlet. Regenerated shoots were cut off from their forming tissue and transferred to Cenkci *et al*. (2008) medium for rooting. After placing the small plantlet lacking rootlet on the modified Cenkci *et al*. (2008) medium for a month, developed roots were formed (Figure 4 (A)).

The difference between the Cenkci *et al*. (2008) medium and the medium used in our study was the consentration of IBA. Whereas Cenkci *et al*. (2008) used 0.1 mg L^{-1} IBA, 2.0 mg L^{-1} IBA was used in present study. There was no observable root formation on control groups which were cultured on a hormone free MS medium. This result is similar to that of Cenkci *et al*. (2008). However, Cenkci *et al*. (2008) analysed auxin pulse and continous treatment of *T. turcica* roots and found that the roots were shorter, thicker, and darker in color after continous treatment. In contrast, our research observed shorter, thinner and pale colored roots that were well-branched (Figure 4 (B)).

A good deal of research has stated that embryo culture can be affected by various factors such as medium component, as well as embryo size, and genotype (Gu *et al*. 1990; Geerts *et al*. 2002; Fratini and Ruiz 2006; Tekdal and Cetiner 2013). The results of this study indicate that the given medium was suitable for the development of roots.

Rooting and Acclimatization: The *in vitro* formed roots in MS medium added 2.0 mg L^{-1} IBA were thick and short, lacking in adventisious roots (Figure 8; 9). During the acclimatization stage of *in vitro* propagation, a few developed plants continued to grow (Figure 4 (C)). After transferring to a pot including streile soil, the percentage of surviving plants did not exceed 5 % due to the fact that the *in vitro* developed roots may have suffered during the transplantation.

In addition, due to transferring the *in vitro* raised plants out of the culture, some environmental stress conditions (water stress, ambient relative humidity, high light intensity, etc.) could have influenced the plants (Bhojwani and Razdan 1996). According to Cenkci *et al*. (2007), potting mixes with soil, perlit, and sand mixture (1:1:1) are suitable for growing of *in vitro* developed *T. turcica* in the greenhouse.

Table 1. A modified culture medium composition for *T. turcica* ovules (Cohen *et al*. 1984).

Culture medium compositions	***Per liter***
Murashige and Skoog salt mixture	4.3 g
Sugars	
Sucrose	100 g
Vitamins	100 mg
Myo-Inositol	
Nicotinic acid	0.5 mg
Pyridoxin-HCl	0.3 mg
Thiamine-HCl	0.1 mg
Gel and other	
Agar	7.5 g
Hormones	
IAA	0.2 mg
GA_3	0.5 mg
Zeatin	0.5 mg
pH: 5.7	

Table 2. *In vitro* embryo culture stages and media

Stages	***Media***
Ovule-Embryo Culture (2 weeks under dark conditions)	Cohen *et al*. (1984) modified medium
Embryo Rescue in Ovule (2 weeks under a 12 h photoperiod)	Cohen *et al*. (1984) modified medium
Regeneration of Plantles (around a month under a 16 h photoperiod)	Cenkci *et al* (2008) modified medium

Table 3. Number of *T. turcica* ovules and embryos cultured on medium and the percentage of plantlets *in vitro* culture

Ovule culture		Embryo sub-cultured from ovule culture	Embryo culture	
No.	No. of viable embryos	No.	No.	Plantlet (%)
50	44	36	34	94

Figure 1: (A) General view of *T. turcica* flower, (B) General view of *T. turcica* fruits and (C) Microscopic view of *T. turcica* fruits

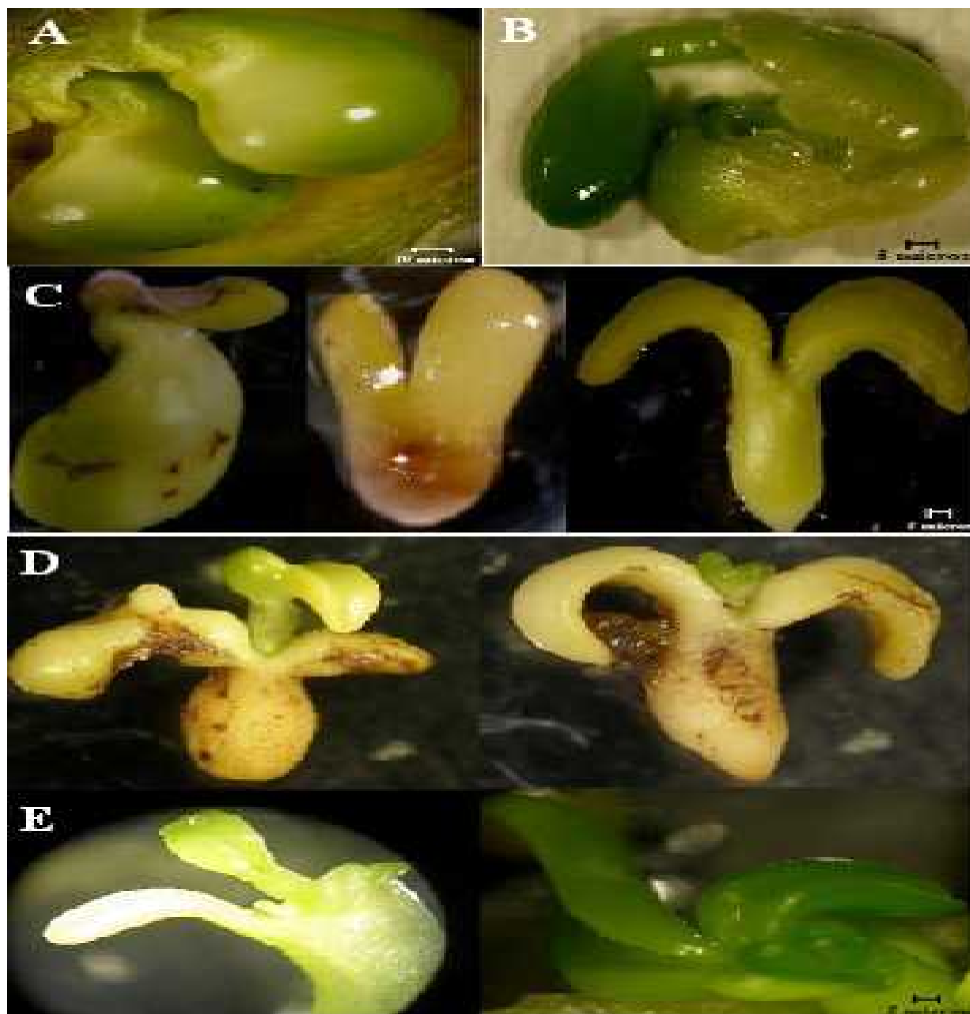

Figure 2: (A) Freshly harvested healthy *T. turcica* fruit seeds, (B) Healthy *T. turcica* fruit seed in coat rupture step, (C) Different embryos at various ages embryos extracted from ovule, (D) Embryos cultured *in vitro* during two weeks

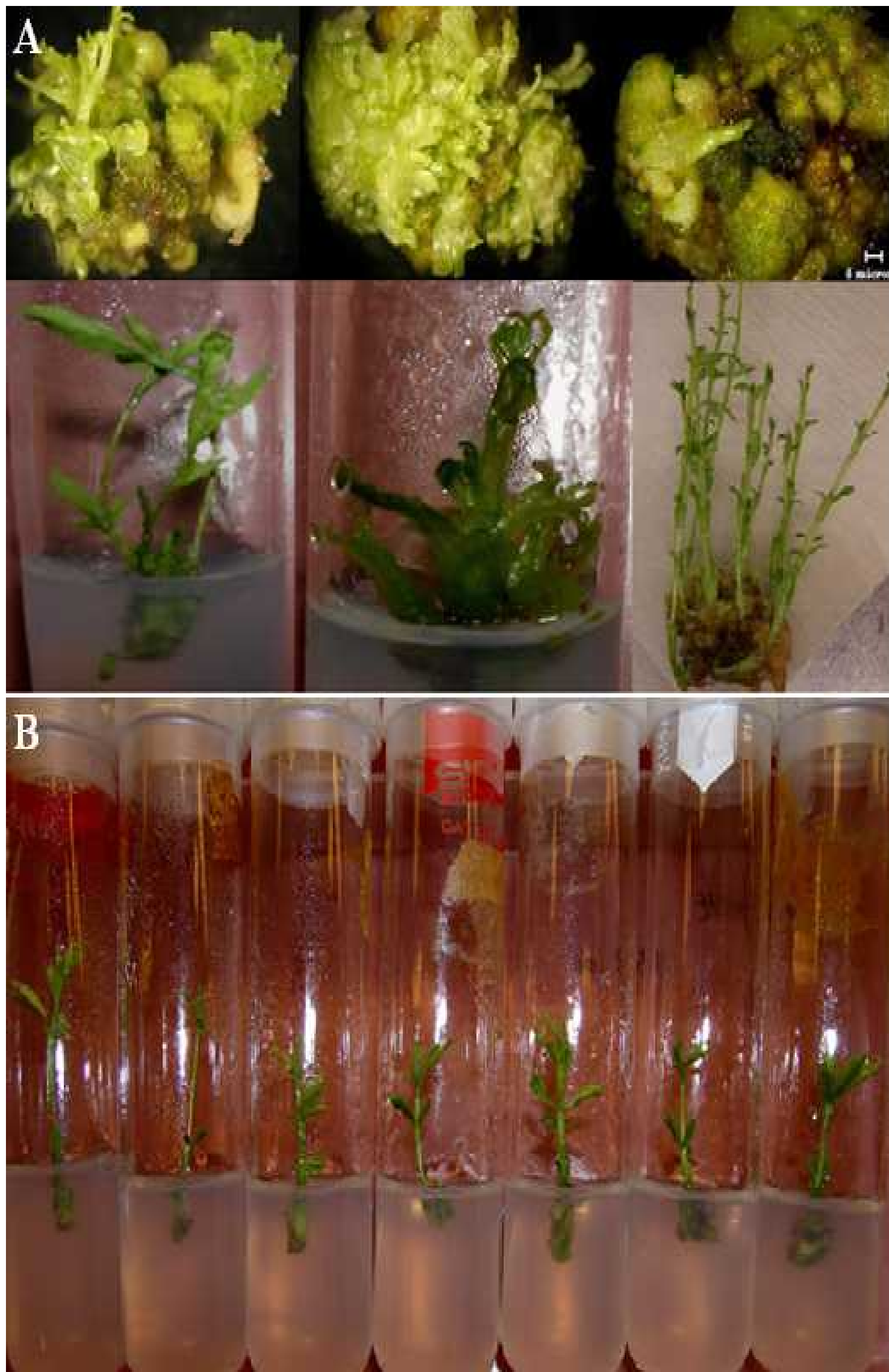

Figure 3: (A) Embryos developed abnormally with induction of multiple buds, (B) Plantlets which lack rootlet after 15 days of culture (tube diameter is 25mm)

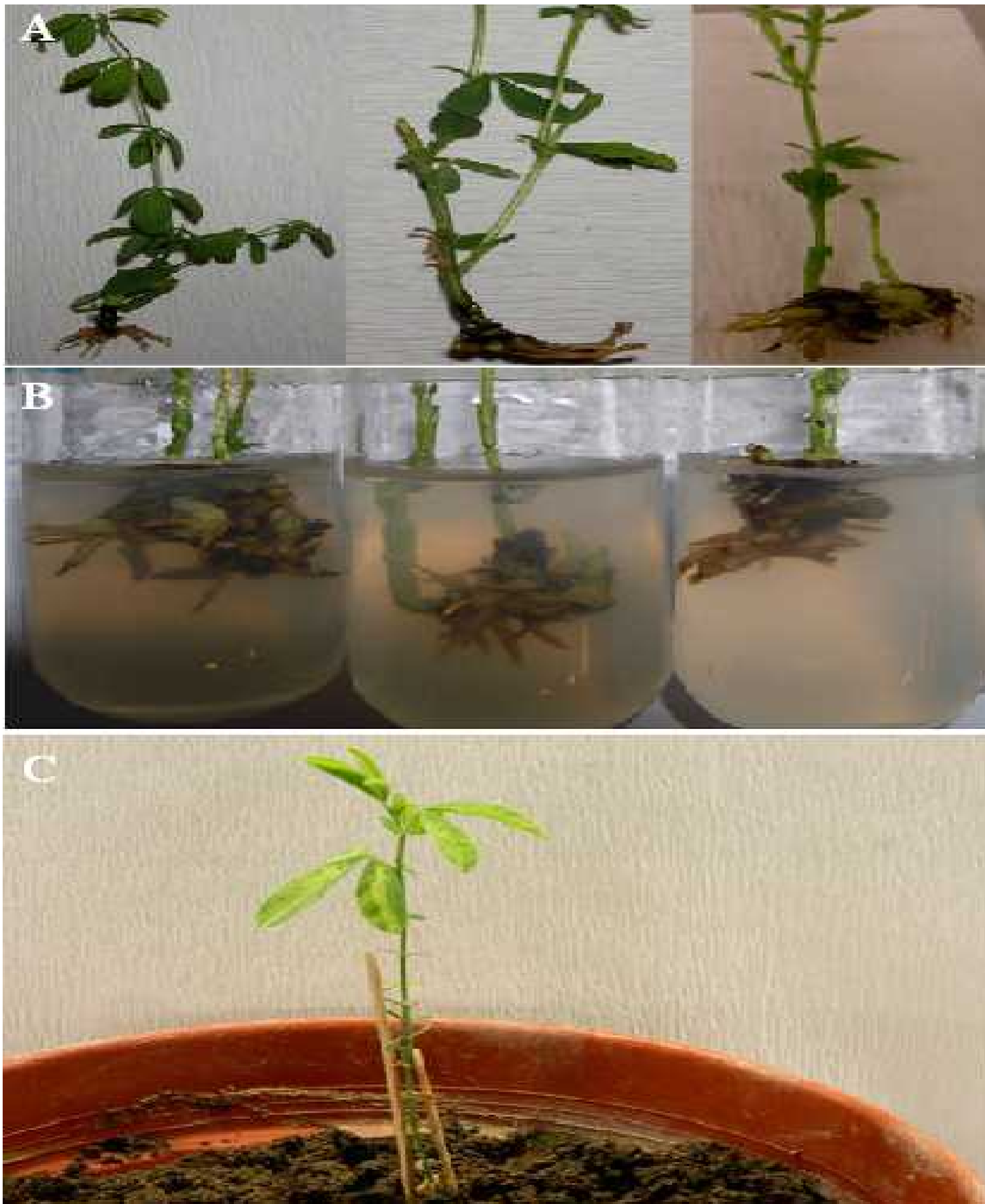

Figure 4: (A) Root and shoot growth in 2.0 mg/L IBA rooting media, (B) The roots of *T. turcica* cultured on MS medium supplemented with 2.0 mg^{-1} L IBA (tube diameter is 25 mm), (C) 5-week-old *in vitro* raised plantlet

Conclusions: Based upon our review of the literature, no embryo culture has to date been performed on *T. turcica* fruit seed. In this study, an efficient embryo culture protocol has been obtained to recover *T. turcica* embryos. In order to increase the number of viable embryos via *in vitro* techniques, future work should entail various hormones and concentrations of different embryo ages. Further studies are needed to understand the germination, physiology, and sexual reproduction of this important endemic, rare Turkish plant. In addition, we recommend that the findings of this study be a source for hybridization and breeding purposes on this plant for future experiments.

Acknowledgement: The authors are grateful to the Nezahat Gokyigit Botanic Garden, of Istanbul, for

providing the germplasm used in the study. We also thank Nancy Karabeyoglu for critically reading this manuscript and providing valuable comments for its improvement. This research partially supported by Yousef Jameel Scholarship Foundation.

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