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INTRODUCING SOME OF THREATENED THYMUS SPECIES TO *IN VITRO* TISSUE CULTURING AS AN APPROACH FOR THEIR CONSERVATION

RAED ALKOWNI[1*], ESRA SOLYMAN[1] AND HASSAN ABU QAUOD[2]

[1]*Biology and Biotechnology Department, An-Najah National University, Nablus, Palestine*
[2]*Plant Production and Protection Department, An-Najah National University, Nablus, Palestine*
[*]*Corresponding author's email: ralkowni@najah.edu; Tel: 00972599794013*

Abstracts

Thymes are considered the most popular herbs in Palestine due to their considerable medicinal and nutritional values. However, at the same time they are also threatened or endangered due to intensive harvesting. Five of the most important thyme species grown naturally in Palestine (wild-types) were collected and their responses to artificial media supplemented with various plant growth regulators had been tested. The results revealed the effectiveness in using the artificial media (MS) with hormonal combinations to regenerate thymes from all five species. The study revealed that callus was induced better with MS media maintained 2mg/L 2,4-D and 0.2 mg/L for all thymes species. Meanwhile media with 1mg/L kinetin and 0.3 mg/L Gibberellic acid were found optimum for shoot proliferation. The best shoot proliferation was recorded for *Thymus incanus* (96%). Developed shoots were easily rooted on media contained 0.01mg/L 2,4 D. Moreover, Organogenesis of thyme plant derived from callus was successfully generated on MS media contained 2mg/L of each NAA and kinetin hormones (1:1 ratio), suggesting additional possibilities of *ex-situ* conservation of these wild herbs. This study was the first to establish a rapid and reliable protocol for micropropagation of wild Palestinian thymes; proposing as well the potential of using this biotechnology in rescuing wild *Thymus* species.

Key words: *In vitro* culture; Biotechnology; Medicinal plants; Plant growth regulators.

Introduction

Palestine is located within the world's nominated "hot spot" areas, where climate changes and human extensive harvesting might be the main reasons for medicinal and aromatic herbs species to be listed as threatened or endangered (Ali-Shtayeh & Jamous, 2002; Abou Auda, 2010). Thymes species were among those listed as threatened plants according to the "Red List of Threatened Plants in the West Bank and Gaza Strip". These perennial herbs of the family *Lamiaceae*, are aromatic plants that have several medicinal and agricultural applications due to their antiseptic properties and traditional supplementary food (Torras *et al.*, 2007). The genus *Thymus* has about 400 species; almost 40 of them were found spread on the Mediterranean region (Daneshvar-Royandezagh *et al.*, 2009). *Thymus* species varied regarding to their morphological features and chemical constitution especially oil compositions. In Palestine, Thyme "Za'tar" is still considered one of the mostly important traditional sources of food; and has a particular importance of people's heritage and culture as an alternative medicine and aroma. The most popular herbs for Palestinian people as food is *Thymus syriacus* Boiss. (synonym *of Majorana syriaca (*L.) Rafin.). Its green leaves are rich of essential oil, which is responsible for its characteristics of flavor and fragrance. Moreover, its extracts were found to have strong biological activity (Al-Mariri *et al*., 2013), due to the presence of phenols, thymol and carvacrol as major constituents of thyme oil in the plant (Abu-Lafi *et al.*, 2007). Another species that are common in the country is *Thymus fruticosus* (L.) Link (synonym of *Micromeria fruticosa* (L.) Druce). That white micromeria has essential oil which largely comprises the monoterpenes (+)-pulegone and isomenthol (Dudai *et al.*, 2001). It is considered very useful herbs for a variety of uses (a refreshing tea, clearing nasal congestion, antiseptic and insect repellent qualities), as well as a cooking spice (Dudai *et al.*, 2001; Baser *et al.*, 1996). *Thymus incanus* (Sm) (synonym of *Calamintha incana* (Sm.) Boiss) and *Thymus capitatus* (L.) Hoffmanns & Link (synonym of *Coridothymus capitatus* (L.) Rchb.f.) leaves containing the essential oil which are strongly used as antiseptic, condiment, deodorant and disinfectant in perfumery, soaps, mouthwash, and flavoring baked goods, beverages, ice creams (Ferrara *et al.*, 2003; Chun *et al.*, 2005; Abu-Lafi *et al.*, 2007; El-Agbar *et al.*, 2008). In addition, *Thymus capitatus* possesses antimicrobial activities which found in its essential oils (Bhaskara *et al.*, 1998; Stahl-Biskup & Seaz 2002; Qaralleh *et al*., 2009.). *Thymus majorana (*L.) Kuntze (synonym of *Origanum majorana*) is widely used in the agricultural, pharmaceutical and cosmetic industries (Chun *et al.*, 2005). They are used in the folk medicine to treat several illnesses as spasmodic, antimicrobial, digestive, expectorant and aromatic for the whooping and convulsive coughs (Dorman & Deans, 2000; Ferrara *et al.*, 2003).

Therefore, these highly valuable medicinal herbs found wild in Palestinians territories are continuously declining. Appropriate measures for their preservation were demanded in the last decades. Modern biotechnology could provide assistances to conserve plants biodiversity (Alkowni & Sawalha, 2012). Besides their ability in producing healthy propagating materials, micropropagation through plant tissue culture is one of the novel technologies to be relied on conserving several medicinal plants. Micropropagation is an important methodology to proliferate homogeneous plants in a relatively short period of time, it is important system of increasing the secondary metabolites productions. Plant tissue culture comprises a wide range of techniques involving the aseptic plant germplasm growth conditions (especially shoot tips, meristems, somatic embryos or embryogenic callus) on artificial media with optimized conditions of culture media, temperature and photoperiod

(Al- Ghabbeish *et al.*, 2006; Leal *et al.*, 2011; Kazmi *et al*., 2013). In an experiment to optimize the tissue culture of *Thymus daenensis* Celak, it was found that the use of 0.5, 1 and 2 mg/l BAP led to increased shoot length, number of shoots and leaf number. The use of 0.5 mg/l BAP and 0.5 mg/l 2,4-D led to highest callus formation percentage (Mirshekar *et al.*, 2014), on the other hand, Hoseini-Beheshti & Khosh-Khui (2006) reported that *Thymus vulgaris* in the presence of 1 mg per liter BA and 0.2 mg per liter NAA showed the highest production of shoot, and 1.25 mg/l of NAA and zero concentration of BA made highest rooting.

Although some common micropropagation steps and protocol were established for many plants and herbs (Paunescu, 2009). However, each species may require specific protocols or optimization. Therefore, this research study was conducted to extend previous trials for conservation of Palestinian plant diversity with developing a robust micro-propagation protocol for rapid multiplication and propagation of wild *Thymus* species to be used in national conservations program in Palestine. Besides, the varied responses of wild *Thymus* species' to artificial media supplied with varied plant growth regulators would be tested.

Materials and Methods

Plant material collection: Several field inspections and investigations were carried out during the winters of 2008-2011, in order to understand the real status of wild thymes in Palestine, five *Thymus* species were selected and identified based on their morphological characters and their properties with the help of taxonomic experts and literature (Solyman & Alkowni, 2014).

Then, the wild types of *Thymus* species [*Thymus syriacus* Boiss (synonym of *Majorana syriaca (*L.) Rafin.); *Thymus majorana (*L.) Kuntze (synonym of *Origanum majorana*); *Thymus incanus* (Sm.) (synonym of *Calamintha incana* (Sm.) Boiss); *Thymus fruticosus* (L.) Link (synonym of *Micromeria fruticosa (*L.) Druce); and *Thymus capitatus* (L.) Hoffmanns & Link (synonym of Coridothymus *capitatus* (L.) Rchb.f.] were collected from several areas in the northern region of Palestine during the year 2010/2011; and maintained in pots under suitable conditions to be used as the sources for *In vitro* culturing (Abu-Qauod, 2004).

Media Preparation and micropropagation: Murashige & Skoog (MS medium -Sigma M5524) was used in all media supplemented with different combinations of plant growth regulators. The solid media was made mainly from 1x MS salt, 3% sucrose, and 0.7% Agar (Sigma A1296), and the pH was adjusted to 5.8 by 1N KOH and 1N HCl before autoclaving at 121°C, 15 Psi for 20 min. Media with PGRs was varied from the purpose that was used for. For callus induction from thyme tissues; MS media was enhanced by various concentrations of hormones auxin (NAA [1-2 mg/L]; 2,4-D [1-2 mg/L]) and cytokinin (BAP [0.1-0.2 mg/L]; Kinetin [0.1-0.2 mg/L]) (Table 1). For shoot induction from callus the used hormones were auxin (NAA [0.1-3 mg/L]; 2,4-D [0.1-3 mg/L]) and cytokinin (BAP [0.1-3 mg/L]; Kinetin [0.1-3 mg/L]). Meanwhile hoot-tip cultures (Proliferation) were induced by using Kinetin [0.1-0.2 mg/L] in combination with Gibberellic acid [0.1-1 mg/L]. For rooting the following hormones were examined: NAA [0.05-0.15 mg/L]; 2,4-D [0-2 mg/L]); and BAP [0.1-0.5 mg/L].

Shooting, rooting and acclimatization experimental conditions: Shoot tips were subjected to chemical sterilization (Murashige & Skoog, 1962) with sterile distilled water, before immersion in 70% ethanol for 1 minute followed by sodium hypochlorite for 5 minutes. Sterilized shoot-tips were cultured on MS media without PGRs (control) and/ or with recommended PGRs combinations MS media. Fifteen replicates were applied for each thymes species and the experiments were repeated twice. All cultures were incubated in a growth chamber at 22± 1 °C for four weeks with 16 hrs of photoperiod illumination of 40 μ mol $m^{-2}s^{-1}$ supplied from cool white fluorescent.

Newly regenerated shoots (3-4 cm length) from shoot-tip media were transferred to MS media with different hormonal combinations of for rooting (NAA [0.05-0.15 mg/L]; 2,4-D [0-2 mg/L]); and BAP [0.1-0.5 mg/L]). Shoots that developed roots were transferred to plastic pots (10 cm diameter) containing sterile media of 1: 1 sand and soil. The cultures were irrigated with sterile tape water and sealed with a thin layer of plastic sheet as recommended (Mikulík, 1999). They were then kept in a growth chamber under the same conditions. After one week, the plastic sheet was removed gradually, and the rooted shoots were then gently transferred into larger pots and kept in a greenhouse conditions. The hardening process was started by preparing sterile media of sand and soil mixture (1:1) and distributed in appropriate plastic cup containers. The transplanted seedlings were irrigated while a plastic transparent cover was placed on the top to prevent dehydration.

Table 1. Experimental design including the PGRs combinations that were used for each stage of micropropagation on thymes.

Stage	Treatment						Optimum treatment
	Auxin	Conc. mg/L	Cyto-kinin	Conc. mg/L	Gibbe-rellins	Conc. mg/L	
Callus induction	NAA 2,4-D	1-2 1-2	BAP Kin	0.1-0.2 0.1-0.2			2,4-D [2mg/L] with Kinetin [0.2mg\L]
Shoot induction from callus	2,4-D NAA	0.1 - 3 0.1-3	Kin BAP	0.1 - 3 0.1-3			NAA with kinetin [2mg/L] at (1:1 ratio)
Shoot-tip culture (Proliferation)			Kin	0.1	GA3	0.1 -1	kinetin [1mg/L] with Gibberellic acid [0.3 mg/L]
Rooting	NAA 2,4-D	0.05-0.15 0 - 2	BAP	0.1 - 0.5			2,4-D [0.01mg/L.]

Results and Discussion

The Palestinian natural ecosystems are hosting relatively large numbers of biota; due to its location on the corridors of the three continents. Global reports have listed more than 51 thousands living species where Palestine's bio-diversities comprises about 3% of that. In fact, more than 2,750 species of plants belonging to 138 families (Danin, 2004), comprising about 149 endemic species (Anon., 2007).

In the last decades and due to human activities in agriculture, traditional food supplements and pharmaceutical health products, urban expanding, and many others; more than 500 species of estimated numbers of plants in Palestine were threatened to endanger. This terrific proportion of threatened vascular plants in the country was alarming to many researchers to get actions in preserving the national heritage of bio-diversities (Ali-Shtayeh & Jamous, 2002; Alkowni & Sawalha, 2012). Therefore, field inspections were carried during the years from 2008-2011 at northern regions of Palestine to reveal significant drop in the wild areas where thyme plants grew. Five of *Thymus* species (*Thymus syriacus* (Boiss); *Thymus fruticosus* (L.) Link; *Thymus incanus* (Sm); *Thymus majorana* (L.) Kuntze; and *Thymus capitatus* (L.)), were collected from their natural habitats and maintained in pots under optimal condition, and their genetic materials and taxonomy were ascertained (Solyman & Alkowni, 2014).

Later on, thymes explants were subjected to *In vitro* culture trials to study their responses to artificial media. Knowing that tissue culture refers to the culturing of all plant parts under aseptic condition (Al- Ghabbeish *et al.*, 2006); selected explants of shoot tips, leaves, seeds, stems and roots were subjected to aseptic conditions by using chemical sterilization. The best results were obtained after several explants soaking with sterilized distilled water (15-20 min) before ethanol treatment (immersion in 70% ethanol for less than one minute), followed by soaking in 20% sodium hypochlorite for 5 minutes. Other changes in sterilization parameters did not give satisfactory results. That might be due to the presence of trichomes on the thymes leaves as well as natural oils that prevent disinfectants from sterilizing plant surfaces comprehensively. Sterilized explants were tested by culturing on MS media without PGRs for a week.

Callus inductions were firstly experimented for thymes explants (leaves and stems) by culturing on solid MS media containing PGRs (NAA / BAP) or (2,4-D / Kinetin) with (10:1) ratios as recommended in previous studies (Alkowni & Sawalha, 2012). Expectedly, callus had started to form in all thymes species within a week, even though the produced masses were varied insignificantly. The experiments showed that MS media which contained (NAA / BAP) stimulated rapid callus formation, but the media with (2,4-D/Kinetin) formed more stable callus with ability to be maintained for long time (8-10 weeks). Referring to that, the callus induction was recommended on MS media that contained 2mg/L 2,4-D as an auxin and 0.2 mg/L Kinetin as cytokinin for all thymes species. This *In vitro* culturing step is considerably important for conservation of plant species genetic materials, or any cryopreservation (Grout, 1995). Moreover, *In vitro* plant cells regenerations were relying on their totipotency capability to initiate somatic embryogenesis (Fehér *et al.*, 2003; Murthy *et al.*, 2010). In addition to that, thymes aqueous culture could be widely applied commercially in bioreactors for production of their valuable compounds as secondary metabolites in biopharmaceuticals (Abu-Lafi *et al.*, 2007; Georgiev *et al.*, 2009).

Stems were directly regenerated into adventitious shoots on cytokinin-containing media with the presence of Gibberellic acid (Fig. 1). The fact that Cytokinins would encourage several shoots to grow from their epicotyl origin, meanwhile Gibberellins (GA) were recommended to enhance shoots and roots elongations (Bidadi *et al.*, 2009). Nevertheless, auxins might be necessary for shoot growth; however, many plants apparently synthesize adequate endogenous concentrations (Trigiano & Gray, 2000; Abbas & Qaiser, 2010). Shoot regeneration started after one week of culture, but not on media without hormones (control). The results of this study were in agreement with Ozudogru *et al.* (2011) who reported an efficient *In vitro* propagation protocol of two *Thymus* spp., results in genetically stable plantlets. Shoot-tip propagation (micropropagation) on MS media with 1mg/L kinetin and 0.3 mg/L Gibberellic acid were found the best, supporting what previous research studies mentioned (Alkowni & Sawalha, 2012). The produced shoot lengths and numbers were used as an indication of multiplication rate for experimented *Thymus* species (Table 2). Expectedly, the results showed differences among the cultured species. Optimum shoot proliferation was with *Thymus incanus* (96%); however only 9.98% of shoots were observed on *Thymus syriacus*. Surprisingly, the same trends were observed with number of shoots for both species (4 times more), meanwhile all species gave almost similar shoot lengths with slight variations. In our study, the *In vitro* variation in the performance of the tested species was in agreement with Shabnum & Wagay (2011), who found different media and hormonal combinations for the micropropagation of *Thymus vulgaris*, *Thymus sipyleus*, *Thymus mastichina*, and *Thymus piprella* species.

Developed shoots were rooted on MS media with auxin concentration ranged from 0 up to 2mg/L; however, they were easily obtained on semi-solid MS medium that was either hormone-free or supplemented with 0.01 mg/L of 2,4-D. Comparative analyses for root lengths were used as arbitrary for selecting the best media. Surprisingly in *Thymus incanus*, and *Thymus capitatus* callus was formed within 3 weeks after shooting, before giving roots after 2 weeks on the same media (MS media with 1mg/L kinetin + 0.3 mg/L Gibberellic acid). On the other hand, shoots of *Thymus majorana*, *Thymus fruticosus*, and *Thymus syriacus* were able to establish direct rooting on MS media without hormone as well as with 0.01 mg/L of 2,4-D. The produced plantlets were subjected to acclimatization process and *ex-situ* conservation trials at relatively low temperature up to 15°C (Fay, 1994). Growth was significantly slowed down, even though more studies on such tale were advised.

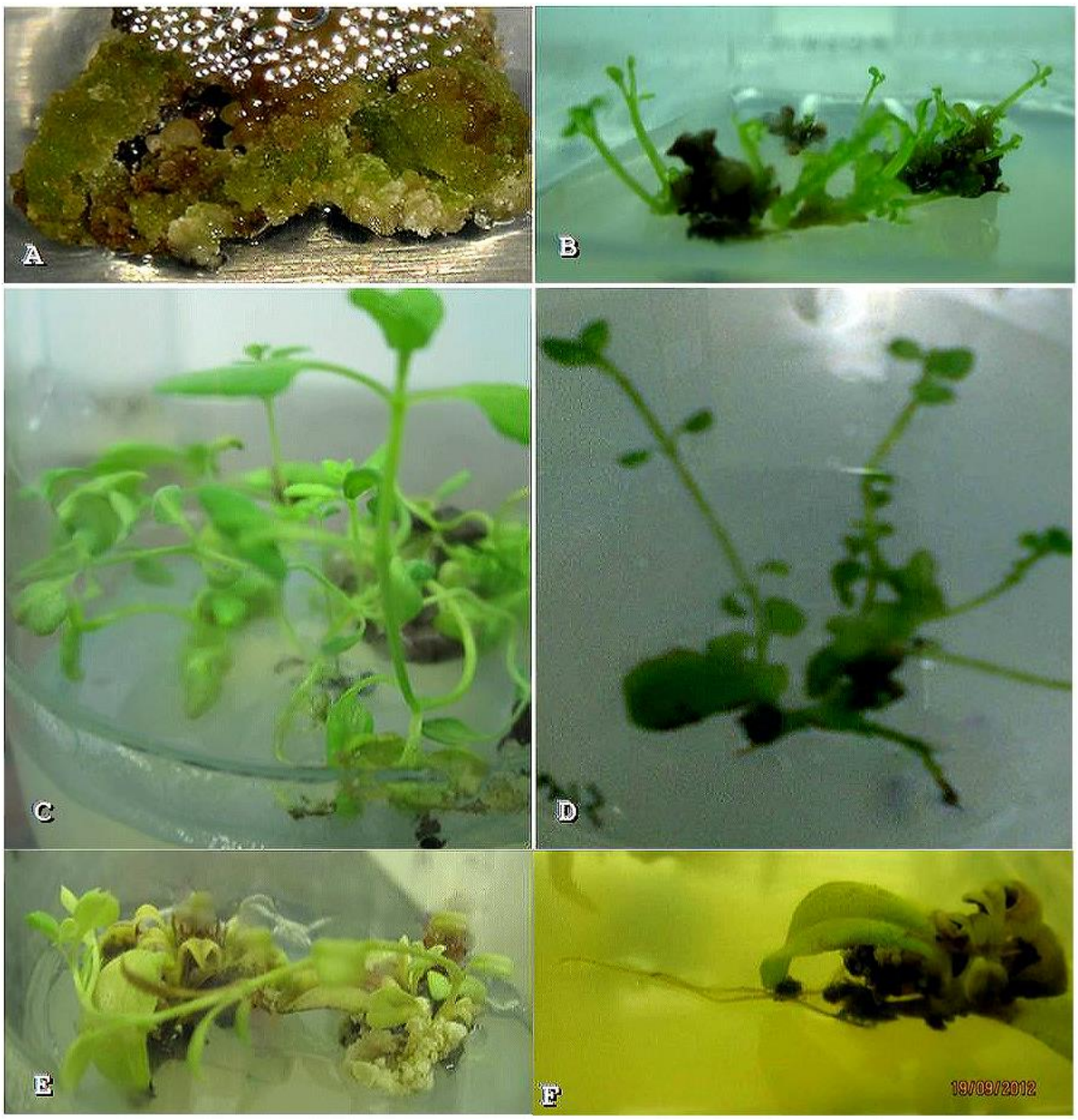

Fig. 1. *In vitro* regeneration and multiplication of *Thymus* species. Callus induction of *Thymus syriacus*(A); Shoots proliferation of *Thymus incanus* (B); *Thymus fruticosus* (C); *Thymus majorana* (D); *Thymus majorana* (E); and rooting of *Thymus incanus* (F), as appeared in this figure.

Table 2. Organogenesis of five thyme species cultured on MS media supplied with kinetin and GA. The percentage of explants producing shoots, number of shoots per explants and shoot length after 5 weeks of cultivation are shown.

Thyme species	Explants producing shoot (%)	Shoot per explant (number)	Shoot length (cm)
Thymus incanus	96.5 ± 4.95	5.41 ± 1.68	5.82 ± 2.07
Thymus fruticosus	74.15* ± 1.20	5.74 ± 1.80	5.68 ± 1.68
Thymus majorana	56.65 ± 4.73	2.93 ± 1.38	3.72 ± 1.35
Thymus capitatus	23.3 ± 4.66	1.57 ± 0.53	4.26 ± 1.01
Thymus syriacus	9.98 ± 4.69	1.33 ± 0.58	3.06 ± 0.85

* Each value represents the average of two experiments ± SD

Organogenesis which occurs either directly or indirectly via callus induction (Trigiano & Gray, 2000), in responses to exogenously added phyto-hormones to regenerate whole thymes plantlets were experimented onto media containing cytokines (Kinetin or BAP) and auxins (2,4-D or NAA) at several ratios. All trials were failed to induce any adventitious shoots from thymes calli except for MS media which contained 2mg/L of NAA and kinetin hormones (1:1 ratio). The results of this study were the first to be carried on wild type of thymes species in Palestine.

It is worth to say that in the last decade biotechnology is used as a tool for conserving plants in general, and medicinal plants in particular (Tripathi & Tripathi, 2003; Alkowni & Sawalha, 2012). Introducing wild thymes species to *In vitro* micropropagation could be served in developing preservation and propagation programs of these endangered *Thymus* species (Sharma *et al.*, 2010; Yadav *et al.*, 2012). This approach for *ex-situ* conservation or germplasm collection for naturally growing thymes in Palestine would be quite useful. Furthermore, *In vitro* tissue culture techniques and storage methods are enabling the establishment of extensive collection using minimum space. Besides establishing *In vitro* plant stocks would have an immediate benefit in reducing the collection pressure on the wild populations. Nevertheless, micropropagated plant materials could be served to respond the market demand on thymes and their products. These collections would allow continuous supplements of thymes propagating materials to wild population for recovery as one of steps towards *In-situ* reservation. Furthermore, having thymes tissues *In vitro* will enable any further molecular investigations, ecological studies, or economical uses.

In conclusion, the *In vitro* culture biotechnology was successfully applied on wild species of thymes, where it would be a promising approach towards rescuing these endangered medicinal plants in the country.

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