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Biotechnology for conservation of palestinian medicinal plants

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Many plants of *Palestinian flora* are facing the risk of endanger, due to agricultural practices, environmental threats and consumption changes. In the absence of National program to conserve the Palestinian heritage of plant diversity, a tentative research work aimed in trial usage of available biotechnology's methods for conserving several popular plants of medical, cultural and economical importance's. Tissue culturing of anise (*Pimipnella anisum*), sage (*Salvia palestina*), fenugreek (*Trigonella sps*), wild peppermint (*Mentha spicita* L.) and akoub (*Gandelia tournefortii*); using MS-media with specific plant growth regulators were successfully applied. Protocols for enhancing callus culturing, organogenesis and micro-propagation of these tentatively threatened wild plants were developed and optimized in this research work. Based on the successfulness of propagation *in vitro* of these plants, a call for establishment of a Palestinian germplasm collection to conserve the *Flora Palestina* had been reported.

Key words: Applied biotechnology, *In-vitro* conservation, Medicinal Plants, micro-propagation, Biodiversity.

Introduction

Palestine, located in the Mediterranean basin, considered as one of the world's biodiversity "hotspot" that should be subjected to conservation (Médail and Quézel, 1997; Myers and Cowling, 1999). *Flora* of Palestine is rich with economical important plants includes vegetables, cereals and fruit trees, providing the local market with essential agricultural crops. More than 2,750 species of plants including 138 families were estimated for Palestinian *flora* (Danin, 2004; Sawalha, 2005). Many of these plants are facing threats of endanger in their natural habitats due to various human population activities such as over-harvesting of wild species, expansion of buildings and the detrimental climatic and environmental changes (ARIJ, 2007). In the last three decades, more than 600 species were reported to suffer from reduction in

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numbers, of which, 90 species are very rare; a cause threat to their existence in the West Bank and Gaza (ARIJ, 2007).

Medicinal plants usage has wide popularity in different Mediterranean countries including Palestine to be alternatives and/or supportive to pharmaceutical drugs (Azaizeh *et al*., 2006). Medicinal plants gain their popularity since they possess natural and bioactive powerful chemical constituents and at the same time they usually cheaper artificially synthesized ones (Halberstein, 2005). Most of these plants are herbs or shrubs grown on rugged mountains and hills, but recently and due to extensive harvesting, some of the most popularly used ones are considered as threatened species (Abou Auda, 2010). As these medicinal plants genetic resources are constantly declining over the years, urgent conservation measures are required as reported by experts (Sultan, 2001). Several approaches could be used for conserving genetic materials, integrates ecology, physiology, molecular biology, genetics, and evolutionary biology to conserve biological diversity at all levels (Campbell *et al*., 2011).

The recent exciting developments in biotechnology have come as a boon as the use of plant tissue culture technique (Sharma *et al*., 2010) or *in vitro* cultivation of plant tissues to serve many fields of applications, especially when producing propagated plants of either hardly species propagated by traditional methods or producing healthy plants free from pathogens (Brink *et al*., 1998; Chaturvedi *et al*., 2004; Alkowni and Srouji, 2009; Sharma *et al*., 2010). In addition to that, investigation on growth and development of plant tissue in culture are more easily controlled than the open field. Micropropagation is amenable to most of economic plants for agricultural, medicinal or cosmetic applications. The differentiation of plant-cultured tissues is controlled by added phyto-hormones to the culture medium in order to develop the whole plant (Salem and Charlwood, 1995). The aseptic technique is highly restricted and culture media are available to make the best growth of biomass for plant regeneration of the plantlets to be then hardened and released to the field.

In this study the main aim was to conserve biodiversity in some Palestinian medicinal plants that are considered as economically and medicinally highly important plant species, through the use of *in vitro* tissue culture biotechnology. In this research work the amenability of the targeted plants for different culturing systems were examined. Since plant tissue culture was considered one of biotechnology tool for conservation of medicinal plants (Trivedi, 2010; Yadav *et al*., 2012), and considering the Palestinian National Authority main goal for promoting the conservation (preservation and sustainable use) of biodiversity in Palestine through appropriate and effective social and economic incentives, this effort which was carried out for the first

time in the Palestine, will be considered as part of innovative approaches leadings for conserving Palestinian plant diversity and establishing a germplasm collection (*ex-sito* conservation) of *flora Palestine.*

Materials and methods

Targeting and Collection of Plant Materials: Medicinal Plants from Palestinian flora that found naturally or imported with social and economical importance in Palestine were collected from wild fields or certified seeds obtained from the local market to be representing to plant-diversity in Palestine. Fresh specimens of targeted plants were collected from fields and/or wild nature during the spring times of 2006-2008. Ex-plant materials mainly stems, leaves or seeds were used in aseptic technique to introduce and establish primary cultures. The following were the popular Palestinian plants targeted for reservation *in vitro*: anise (*Pimipnella anisum*), sage (*Salvia palaestina*), wild peppermint (*Mentha spicita* L.), akkoub (*Gandelia tournefortii*), and fenugreek (*Trigonella sps*). Selected explants were shoot tips, leaves, seeds, stems and/or roots. These explants were subjected to aseptic conditions by using chemical sterilization (ethanol and/or sodium hypochlorite) for different periods of time.

Media Preparation: Culture medium could be as a liquid or a gel, designed to support growth of cells. MS media was developed in 1962 by Murashige & Skoog, and made of compound of mineral salts, many vitamins and other energy resources. Murashige & Skoog (MS) media with different combinations of plant growth hormones were extensively used in this study. Based on our previous experience, the media constructed from MS salts 4.4 g L^{-1} and sucrose 30g L^{-1}, with or without plant growth regulators (PGRs) (depending on culturing type and plant stage) were found the best one for establishing all cultured media (unpublished data). 1-3% Agar was added to solid medium.

Shoot Culturing: Clonal propagation techniques (*Micropropagation*) using shoot tip and nodal segments as the main strategy for *ex-sito* conservation were applied to multiplication and conservation of collected plant species. Ex-plants introduced to tissue culture were sterilized using chemical detergents with rigid aseptic conditions. The principle of shoot-tip propagation is to multiply shoot tips by culturing them on cytokinin-containing media to encourage several shoots to grow from the original. Auxin also may be necessary to obtain shoot growth; however, many plants apparently synthesize adequate endogenous concentrations. Shoot-tip culture was also very important in propagation as the technique used to get rid pathogens from clones. In this case the smaller the shoot-tip cultured the better the chance of propagating plants free of virus, bacteria or mycoplasma. Frequently, true meristem culture is employed for this purpose (Pierik, 1989). In these experiments MS media with different PGRs

(GA_3, BAP and Kinetin) combinations were used. Concentrations of exogenous plant hormones added to the MS medium in shoot culture were BAP combined with GA_3 range from 0.1- 0.5 (increase by 0.1) mg L^{-1}. In other trials Kinetin was added to these combinations.

Regeneration and Hardening Process: Rooting of newly formed shoots were achieved in different media with or without different auxins concentrations. 2,4-D and NAA, were the only auxins used. Acclimatization of the newly produced plantlets by plant tissue culture was processed for hardening at lab scale, followed by green house level. Having produced regenerated plants (plantlets that had 2-3 leaves and one strong root) from tissue culture, the hardening process would be started by preparing sterile medium of sand and soil mixture (1:1) to be then distributed in appropriate plastic cup containers. The plantlets were removed from artificial medium and rinsed with sterile water to remove excess of agar on roots, to be then placed into the cup, introducing the roots into the soil sand mixture. The newly transferred plantlets were spray-irrigated while they stilled under transparent plastic cover on the top to prevent quick dehydration. The regenerated plantlets were placed in growth chamber (room) for one week, before they were transferred to screen house for several weeks, with gradual release of plastic covers.

Callus Induction: In plant biology, callus is the cells that cover a plant wound (Chawla, 2002), while in biological research and biotechnology, the callus is undifferentiated, slowly dividing and growing mass of cells in particular conditions. Hormonal supplements (mainly auxin and cytokinin balance) were adjusted according to criteria depending on plant species and environmental conditions applied. In our experimental trials, callus was produced by placing part of plant tissues (leaves, stems, roots, and seeds) in conditions that induced them to begin dividing. The times needed were varied depending on explants kind, culture conditions, and their response to PGRs added to the media. The different PGRs combinations (2,4-D, NAA, Kinetin and BAP) had been used in MS media. Callus inductions were achieved using 2,4-D combined with kinetin range from 0.6 – 2.0 or 4.0 (increase by 0.2) mg L^{-1}. In other trials NAA combined with BAP were replaced the previous ones.

Later on Callus cultures were used organogenesis and /or to establish other cultures such as suspension one. Plant cultures were maintained and sub-cultured to conserve plant stock *in vitro*.

Organogenesis: Organogenesis can occur in two ways: *direct organogenesis* using roots to produce shoot, or shoot to produce root, with no callus intermediate, and *indirect organogenesis* by using the callus as an intermediate for rooting or shooting enhancements (Lombardi *et al*., 2007; Pal *et al*., 2007). Organogenesis happens in various plant tissue cultures in response to

exogenously added phyto-hormones or under the effect of environmental conditions. The auxin/cytokinin ratios considered vital in root/shoot enhancement for several plants (Chawla, 2002). For example, the media with high auxin usually induce root formation, while shoot formation would be induced with low auxin/cytokinin ratios. Regeneration of shoots and/ or roots from callus was carried by using several experimental MS media with different PGRs (2,4-D, Kinetin, BAP, and NAA) combinations range from 0.1- 4 (increase by 0.5) mg L^{-1}.

Cell suspension culture: This process is particularly suitable for physiological, biochemical, and molecular studies as well as for the process of somatic embryogenesis. The best culture media were the media used for callus induction without solidifying agent. Cell suspension culture was established for each plant in this study research, enhancing single cells or cell aggregates to proliferate in liquid media. To ensure good culturing system, a growth curve for each culture was drawn using packed cell volume (PCV) (Falco *et al*., 1996). Briefly, homogenous sample of the culture was removed and centrifuged. The pellet then can be determined as ml pellet per ml culture.

Results

Collection and Sterilization of Plant Materials: Plants had been selected to be representative to Palestinian *flora* medicinally, culturally and economically important to Palestinian people. Some of these plants were also considered endangered and have heritage value. A total of five different plant species were collected from fields and/or wild nature were subjected to be as representative examples for *ex-sito* conservation. In order to get clean ex-plants for tissue culturing, seeds from some of these plants were germinated successfully *in vitro as in Fenugreek and Anise* (Fig. 1) after restrict sterilization process. Either seeds or ex-plants were subjected to several trials of soaking in sterilizing solutions, with different periods of time, before applying them to culturing media. To overcome the external contaminants, several trials were made to infer the best procedure ensuring minimal contaminations and keep plants viable. In our hands, we found the best results by soaking ex-plants in ddH_2O for 30 min prior dipping them in 10% of Ethanol solution for just 1-2 min followed by several rinsing with ddH_2O. They were then immersed in 0.5-1% sodium hypochlorite solution for 25 min, followed by three times rinsing with ddH_2O, 10 min for each, and most importantly to be carried under hood. Ex-plants were then semi-dried on autoclaved clean tissue-papers by leaving them for several minutes and always under Laminar flow hood. The ex-plants were then ready to be processed for *in vitro* culturing.

Shoot Culture: Micropropagation (shoot culture) provides solution for mass propagation of plants in particular for those considered as endangered ones for their conservation within short period and limited space. The plants produced from this method should be true to type. Sterilized shoot-tips were cultured on several media containing plant growth regulators at different concentrations. It was noticed that different plant species varies in their responses' to the media. Using combinations of PGRs with MS media as abovementioned gave various growth and proliferations depending on the type of plants used. Adding GA_3 with either cytokinin hormones significantly increased shoots length in short period of time (un-shown data). However, satisfactory results were obtained by culturing the shoots in the media that contain 4.4g L^{-1} MS salt, 30g L^{-1} glucose (pH 5.7), 5.7g L^{-1} agar, 0.1mg L^{-1} kinetin, 0.1mg L^{-1} GA_3. Increasing the kinetin more than that showed retardation in shoot proliferation. While using BAP has no differences in proliferation of all plants used in this study, except for sage. Using BAP instead of kinetin was found much favorable for culturing common sage. Proliferated shoots were excised and planted on several media for enhancing rooting. It was found to be as best as in MS media -zero PGRs. Full and half strength were tested to reveal that full length had little shorter roots, but larger in size. These results were evaluated to cluster with no significant differences if compared with those rooted in MS-media with 2,4-D or NAA (Fig.2).

For example anise (which was initially started and studied intensively), shoot cultures were obtained following the transfer of previously induced callus cultures to MS medium without adding hormones. The general characterization of the shoots is emerging from a biomass of callus, i.e. shoot differentiating callus. The later culture was used to establish suspension culture of *P. anisum*. The degree of callus differentiation was largely dependent upon the type and concentration of phytohormones added to solidify MS medium. The results showed that maximum production of anise shoots (52% of fresh biomass) were at combination of 5 mg/l BAP and 1 mg L^{-1} NAA (Fig.2). This was true for sage (Fig.3). When the concentration of NAA was at higher concentration compared to BAP, the effect was dramatic reduction of shoot production reaching 13.8 % of fresh biomass and leading to differentiated roots, (Table1) anise

Table 1. The percentage of biomass in anise cultures grown under light conditions on solidified MS medium for 5 weeks

BAP/NAA	Shoots	Roots	Callus
Ratio	%	%	%
5/1	52.0+15.7	1.8+0.4	46.2+15.8
1/1	6.9+2.7	2.5+0.6	90.6+2.5
1/5	13.8+3.9	4.2+2.4	82.1+4.7

Rooting was easily achieved by using MSO media. The effect of Auxin (i.e 2,4-D were observe in boat shaped root (thickened and shortened roots, once the hormone concentration increase from 0.05 to 2mg L^{-1}. (Fig. 4).

Acclimatization process was achieved on small scale for some plants and the following the previously mentioned procedure was found very successfully in hardening micropropagated plantlets. After one week of transferring plantlets into nursery soil mixture (mainly *peat moss*), the plastic covers were removed partially to reduce internal humidity and allowed for further acclimatization. Two weeks after transplanting, the covers are removed entirely and plantlets are exposed to full sun light. The soil was kept wet and diluted fertilizer was added. After a month of transplanting from culture, the plantlets were strong enough to be transferred to new pots with normal Micropropagation process (from shoot proliferation till the end of acclimatization stage) which took a time from 8 to 12 weeks.

Callus Induction: Using different media for inducing callus had been tested intensively with variable media concentration. All tested plants did not induce any callus within 3 weeks after transplanting, except in the MS media which containing auxin with cytokinin with a ratio of 10:1. In about 10 days, the best PGRs to induce callus were found (2mg L^{-1} of 2,4-D and 0.2 L^{-1} of kinetin), and stimulate callus formation rapidly. The undifferentiated cells divided very fast without any control but start dying after the 3rd week. NAA & BAP were also tested for their callus induction to be found less effective compared with 2,4-D and Kinetin contained MS-medium (Fig.5).

The growth of initiated callus was slow at the beginning reaching 5mm diameter of spherical shape, but the callus was maintained for further subculture onto same medium every 4th weeks. However, callus initiation was more successful and faster when exogenous phytohormones were added to MS medium at combined concentration of 0.2 mg L^{-1} kinetin and 1 mg L^{-1} 2,4-D.

The Callus cultures of all tested plants were established and maintained every month. The most ideal ex-plant for establishing cultures was found the stem for all examined plants. It is worth to mention that some plants such as *G. turnifortii* found to be recalcitrance (Fig.5). This may due to their nature to

grow in cold conditions that it was adapted to. Some plants as *Salvia palaestina* were found difficult to sterilize, due to large density of trichomes on its leaf surface.

Organogenesis: After a month of callus formation, organogenesis had been tested for plants. As direct organogenesis had been tested in micropropagation, indirect one had been applied by culturing pieces of callus on MS-media containing BAP with different PGRs (2,4-D, Kinetin, BAP, and NAA) with combinations ranges from 0.1- 4 (increased by 0.5) mg L^{-1} to induce either shoots or roots. The experiments revealed that the best response was obtained by using MS media with 2mg L^{-1} NAA and 2mg L^{-1} kinetin and only for sage shoot formation (Fig. 3), while failed for akkoub.

Cell suspension culture: The mass of the callus were cultured in a liquid MS media (without agar) that contain the same PGRs used for callus induction (2mg L^{-1} 2.4D and 0.2 mg L^{-1} kinetin), and the rate of their growth had been measure every week. The growth curve had been drawn for each suspension (batch) culture using the packed cell volume (PCV) method. The growth rate of suspension cultures were found faster than calli ones.

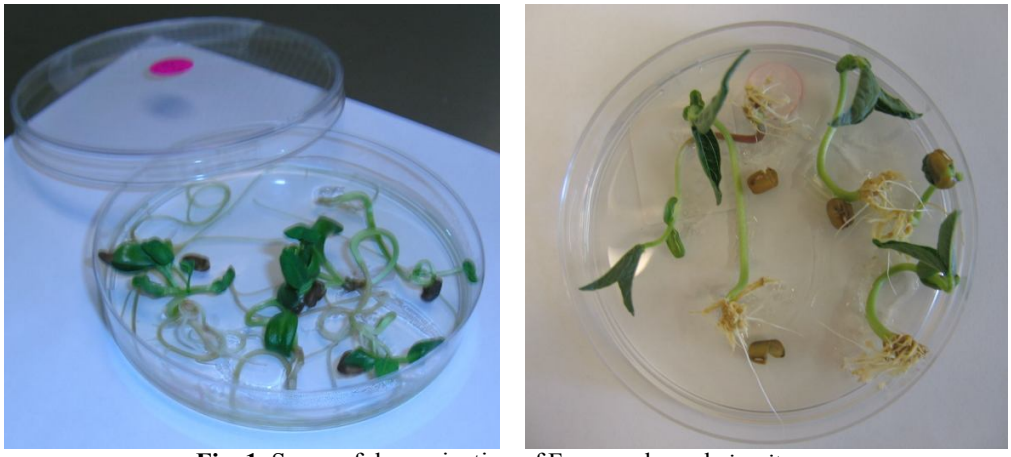

Fig. 1. Successful germination of Fenugreek seeds *in vitro*

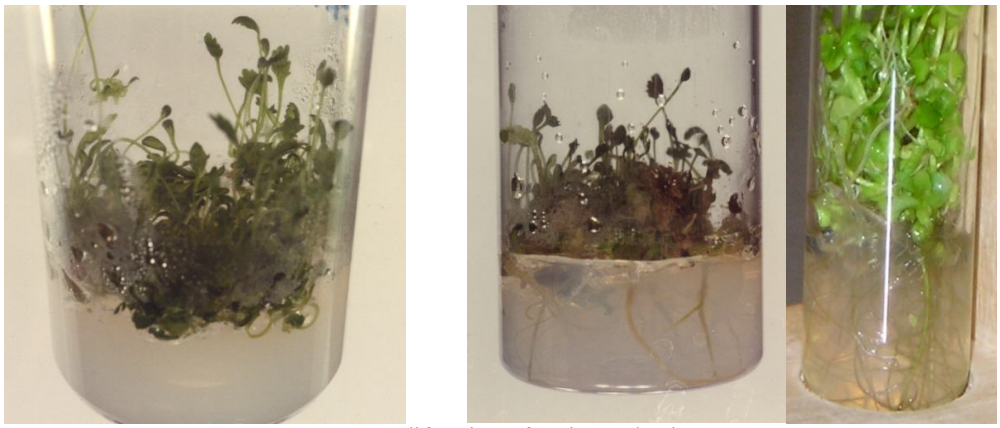

Fig. 2. Proliferation of Anise and Mint.

Fig. 3. Soot formation of Sage

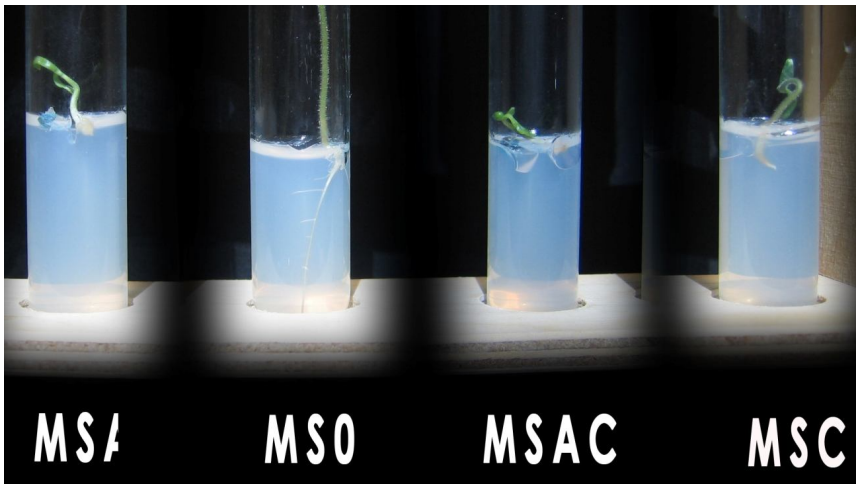

Fig. 4. Roots formations were inhibited by adding PGRs. The best rooting was found in MS-media with absence of any PGRs. From left to right, MS-media with Auxin, without hormones, with Auxin & cytokinin, and last one MS media with cytokinin phytohormone.

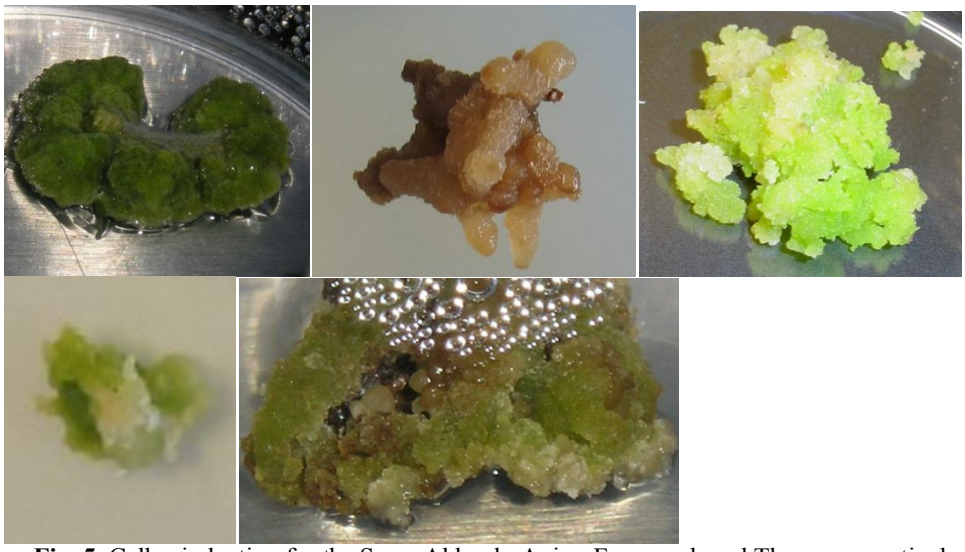

Fig. 5. Callus induction for the Sage, Akkoub, Anise, Fenugreek and Thyme respectively.

Discussion and conclusions

The Mediterranean Basin has a long history of land conservation. As early as 2,000 years ago, the Romans and Greeks set aside areas for the protection of natural resources. Palestine is part of the Mediterranean biodiversity hotspot including more than 51,000 living species in (ARIJ, 2007), comprises about 3% of the global biodiversity, where plant diversity is composed of an estimated 2,750 species of plants in 138 families (Danin, 2004).

The over-exploitation of plants is eroding genetic resources in Palestine (Vié *et al*., 2008). In Addition to that, pathogens and pests, as well as unplanned expansion or industries, were leading for disappearance of the most species and/or varieties as a result of that (Alkowni and Srouji, 2009). Since terrestrial and aquatic biological resources are seriously threatened (primarily) by human activity and the growing threat of climate change, seek for conserving Palestinian diversity become crucial. *Flora Palestina* are one of the potentially important economically, medicinally, culturally, and overall members of global biodiversity. Olives, Sage, Thyme, Akkoub, etc, are considered as national cultural heritages beside their vital role in economy and medicinal usage (Azaizeh *et al*., 2006). Meanwhile, these Palestinian species (so far called "*Baladi*") are red listed to be threatened due to building expansion, imported or introduced varieties and cultivars, climate change and over usage (ARIJ, 2007).

While biotechnological applications can be performed for human benefits, it is worth to be used for conserving plant diversities. *In vitro* tissue culture is proved as viable technique to be used as a tool for *ex-sito* conservation of plant diversity and mass production by micropropagation (Chaturvedi *et al*., 2004; Droste *et al*., 2005; Al-Gabbiesh *et al*., 2006; Hoffmann *et al*., 2008; Kereša *et al*., 2009; Paunescu, 2009; Cristea *et al*., 2010; Sharma *et al*., 2010).

This study was aimed to apply *in vitro* culture as one of biotechnological tool for rescuing Palestinian plants, in particular medicinal one's, an approach had been proposed by several researcher abroad as an effective tools for conserving biodiversity (Tripathi and Tripathi, 2003; Paunescu, 2009; Sharma *et al*., 2010; Trivedi, 2010) and used for mass propagation of various medicinal plants.

Several plants were tried to be as part of trials for implementation of *in vitro* culturing and micropropagation. We reported here only 5 different medicinal plant species: anise (*Pimipnella anisum*), sage (*Salvia palaestina*), wild peppermint (*Mentha spicita* L.), fenugreek (*Trigonella sps*) and akkoub (*Gandelia tournefortii*) that wew tested for their phytohormonal responses to regeneration. These plants should be considered as initial start for what we proposing as "National program for *ex-sito* conservation of *Flora Palestina* (Germplasm collection)". Using tissue culture techniques with different hormonal balances and combinations were proved as vital parameter for culture initiation. As some of the plants used in this study were experimentally tested for the first time with different hormonal combinations and /or the technique were optimized. For example, Anise (*P. anisum*) seeds, that extensively studied and used as a model for other medicinal herbs, were germinated under aseptic technique conditions to give 80% germination rate on MS medium without addition of phytohormones (Fig. 1), while the mature seedlings (after 2-3 weeks old) were then used to establish a callus culture, which erupted from seedlings directly. Callus induction was achieved by MS medium combined with 0.2 mg L^{-1} kinetin and 1 mg L^{-1} 2,4-D. Organogenesis (shoot) were obtained directly from induced callus cultured in MS media without PGRs at the beginning, followed by sub-culturing media with combination of 5 mg L^{-1} BAP and 1 mg L^{-1} NAA (Fig. 3).

The use of *in vitro* methods considered as a rapid propagating tool, capable of producing a large number of plant in a relative short time (Herris and Stevenson, 1982; Monnette, 1985). First attempts to harness *in vitro* methods for this purpose were initiated in the 1960s by Glaze (Glazy, 1972). The advances of tissue culture technology enable now production of virus-free material via meristem tip (Barlass *et al*., 1982; Roca *et al*., 1989; Barba *et al*.,

1990; Nehra and Kartha, 1994), fragmented apexes (Barlass and Skene, 1980), and *in vitro* somatic embryogenesis (Goussard and Wiid, 1990). In these cases, *in vitro* methods are combined with chemotherapy or thermotherapy (Barba *et al*., 1990) by which plants acquired can be rapidly propagated using *in vitro* culture.

Germplasm conservation is essential for maintaining genetic diversity and gene pools for crop improvement. Field maintenance of woody plants, which cannot be stored as seeds, is expensive, demands huge areas and labor and encounters many pest problems. Using conservation in tissue culture applying various slow growth techniques, cryopservation are of great benefit to these crops (Kartha *et al*., 1989). Genetic manipulations and transformation open a new field in plant breeding. Conventional breeding uses *in vitro* techniques such as embryo cultivation and regeneration, protoplast fusion anther culturing. The ability to introduce foreign genes into plant cells using efficient vectors and regenerating viable plants, opens new opportunities for breeding improved plants, modifications etc. (Gasser and Fraley, 1989). These methods are now experimented aiming to produce virus resistant plants by introducing of different virus genes.

Recently, the significant advancement of biotechnology is available to conserve the threatened plant germplasm. The new developed techniques offer new options and facilitate conservation in the form of seeds, pollen, embryos, and *in vitro* cultures. (Paunescu, 2009). Establishing *in vitro* plant stocks have an immediate benefit by reduction the collection pressure on the wild populations. These collections allow for continuous supply of valuable material for wild population recovery, molecular investigations, ecological studies, or economic uses. Various aspects of plant science, both basic and applied, have benefited from the application of tissue culture technology such as: breeding programs for plant improvement, disease elimination, clonal propagation (micro-propagation), cells behavior studies and germplasm storage. Micropropagated plants will be sources for genotyping studies and molecularly identification of each plant.

Moreover, the ability to had suspension culture for each medicinal plant will be potentially sources for further biochemical studies. In this tale, the value of applying *in vitro* cultivation of medicinal plants will extend beyond the rescue of threatened species, within a conservation program to the ease of production and use of secondary compounds which can be served as pharmaceuticals, food additives, bio-pesticides or even as perfumes.

The steady increase in Palestinian population similar to that of other developing countries population; accompanying with an increase of their activities, had affected the natural habitat for a great number of herbs and trees.

Beside that some of Palestinian wild plants are facing extinction due to overexploitations. To conserve that genetic treasure of the country, a rapid biotechnological tool such as micropropagation will be inevitable. Nowadays, *in vitro* culture has emerged as a promising technique to obtain genetically pure elite populations under *in vitro* conditions rather than have indifferent populations. The success of establishing a protocol for culturing 5 Palestinian medicinal plants *in vitro* had been proved in this research and recommended to be extended for other plants.

This study proved and recommended for the first time the use of *in vitro* culture to conserve flora of Palestine. We had succeeded to conserve some of endangered and/or threatened plants *in vitro*, and we recommended this approach to be extended to other Palestinian *flora*. Finally, we propose to establish a germplasm collection for *flora Palestinae* where *in vitro* culture will be used as a best tool of choice.

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