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Study of callus induction in wild *Salvia fruticosa* Mill. from Al-Jabal Al-Akhdar area (Libya)

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Abstract
An *in vitro* cultivation protocol was developed for *Salvia fruiticosa* Mill, a species threatened by over collection due to its importance as a medicinal plant in Al-Jabal Al-Akhdar area, Libya. The aim of this study was to develop an efficient micropropagation method for. *fruiticosa,* that would contribute to the conservation and commercial propagation of this species. Effect of different plant growth regulators onCallus induction, explants (leaves, stems and roots) cultured on MS media supplemented with: Dichlorophenoxyacetic acid (2, 4-D), Benzylaminopurine (BAP) and Naphthaleneacetic acid (NAA). Interaction between media composition and types of explants (leaves, stems and roots) on callus induction %, fresh /dry weights and water content showed that leaf explants responded with higher significant levels (70%, 2.841, 0.186 g/jar and 60%) respectively then roots (62%, 1.321, 0.034 and 33%) and lastly, stems (22%, 0.123, 0.031 and 16%) when cultured on medium received only 2,4- D.

Keywords: callus induction, micropropagation, *Salvia fruticosa*, tissue culture

Introduction

Al-Jabal Al-Akhdar region lies on the Mediterranean coast of northeastern Libya (Cyrenaica) which is known as (The Green Mountain, GM); a three terraces hill that raises gradually, the highest terrace reaches about 880 m above sea level. Al-Jabal Al-Akhdar has a distinctive moderately large biodiversity, especially in valleys and depressions. Libya is considered to be rich in medicinal plants, especially Cyrenaica which has the majority of all medicinal plants documented in Libya (El-Mokasabi, 2014; El-Mokasabi *et al*.; 2018) [14, 13].

Similar to other plant species in Libya, Medicinal plants endure from severe biodiversity devastation and degradation especially along the Libyan coast (Louhaichi *et al.,* 2011; Agiel & Mericli, 2017) [22, 2]. From these cureless and distracting activities which cause damage to several habitats and communities disturbance are: (overgrazing, ploughing, land reclamation, urbanization, mining, forest fires, over-collecting, and charcoal production). Consequently, hundreds of species are threatened with disappearance where many are at the edge of extinction (El-Barasi & Saaed, 2013) [12]. Population growth, urbanization and the unlimited assortment of medicinal plants from the wild lead to an over-exploitation of natural sources. Therefore, there is an urgent need to manage traditional medicinal plant resources (Sharma *et al*., 2010) [34].

Vast amounts of money are consumed each year to replace vanished biodiversity, and many protocols have recently become available. Unfortunately, there is no natural improvement in the condition of these plant species, and the number of endangered plant species is slowly but steadily increasing (Tripathi, 2008). Reproduction and conservation of plants using *in vitro* culture is one of the viable methods to protect rare and endangered taxa (Maryam *et al*., 2014) [25].

There is a lack of information regarding the medicinal plants in Al-Jabal Al-Akhdar region including their autecology, distribution, productivity and possibility of cultivation. It is crucial to carry out studies on these plants and examine conservation methods. The main concern of plant diversity conservation in this district is to concentrate on medicinal, rare and endemic species, by focusing on species that are significant to the ecosystem, for instance, *Arbutus pavarii*, *Pinus halepensis*, *Querecus coccifera*, *Juniperus phoenicea*, *Olea europaea*, *Ceratonia siliqua*, *Pistacia lentiscus*, *Ziziphous lotus*, *Myrtus communis*, *Rosmarinus officinalis*, *Thapsia sylphium*, *Thymus capitatus*, *Salvia fruticosa* and *Artemisia herba alba* (El-Barasi & Saaed, 2013; El-Mokasabi, 2014) [12, 14].

In Libya, *Lamiaceae* family showed the highest number of medicinal species (8 species), which are: *Phlomis floccosa* D. Don, *Rosmarinus officinalis* L., *Marrubium vulgare* L., *Satureja thymbra* L., *Salvia fruticosa* Mill., *Thymbra capitata* (L.) Cav., *Teucrium polium* L. and *Ocimum basilicum* L. These formed 9% of medicinal plant species found in Al-Jabal Al-Akhdar area (Mukassabi *et al*., 2017) [26].

Salvia species is considered as one of the largest genera in the *Lamiaceae* family and includes around 1000 species that have the most international distribution (Cvetkovikj *et al*., 2015) [10]. *Salvia* species have been used in traditional medicine all around the world since ancient times (AL sheef *et al*., 2013) [5].

There's, an increasing awareness within the advancement of proficient conventions for the tissue culture and micropropagation of certain *Salvia* species (Kintzios, 2000) [19]. Therefore, we selected *Salvia fruticosa* Mill for this research because it is one of the most important medicinal plants in the region which is lacking a lot of information.

Salvia fruticosa Mill. (syn. *Salvia triloba* L.) or three-lobed sage (syn. Greek or Turkish sage), or mariamia as known in the Middle East, is an interesting medicinal and aromatic shrub belongs to family *Lamiaceae* (Abd El-Wahab *et al.*, 2015). This species is indigenous in the eastern Mediterranean spread from Italy, Sicily, and Cyrenaica, through the southern Balkan Peninsula to western Syria (as cited in AL sheef *et al.*, 2013) [5].

S. fruticosa utilized in traditional medicine and appreciated for its beauty, medicinal characteristics, culinary usage, together with its sweet nectar and pollen (AL sheef *et al.*, 2013) [5]. This herb (especially the leaves) is also used for the treatment of a range of skin, blood, and infectious illnesses in addition to diseases of the digestive, circulatory, respiratory, and osteomuscular systems (Ali-shtayeh *et al.*, 2000; Carmona *et al.*, 2005) [6, 9]. Furthermore, this species has a hypoglycemic effect and can be utilized against inflammations, hepatitis, and tuberculosis (Pitarokili *et al.,* 2003) [31]. The essential oil from *S. fruticosa* has antimicrobial properties (Pierozan *et al.*, 2009) [30]. Also, Pitarokili *et al.,* (2003) [31] reported that this herb has antifungal activity. There are many studies conducted on the chemical composition, antimicrobial and antioxidant activities of the essential oil of this species (Giwelii *et al.*, 2013).

Biotechnology techniques are important tools for the conservation of medicinal plants. Due to extensive collection of *S. fruticosa* from the wild in Al-Jabal Al-Akhdar area, conservation of this plant species through *in vitro* regeneration is essential. Limited information regarding regeneration protocols in *S. fruticosa*, therefore our main goal was to develop a protocol for micropropagation of this important medicinal plant.

Plant tissue culture is the propagation of a plant by using a plant part or single cell or group of cells in a test tube under very monitored and sterile conditions (Sharma & Dubey, 2011) [35]. A single explant can be multiplied into several thousand plants in a comparatively short period of time in a continuous process (Akin-Idowu *et al*., 2009) [3]. Plant tissue culture has been extensively used in agriculture, horticulture, forestry, and plant breeding areas. It is an applied biotechnology utilized for mass propagation, virus exclusion, secondary metabolite construction and *in-vitro* plant cloning (Oseni *et al*., 2018) [29]. Recently, more attention has directed to in *vitro* culture of plant cells and tissues as it offers the ways to investigate the physiological and genetic processes of plants besides providing the ability to aid in breeding improved cultivars increasing their genetic variation (Lutts *et al*., 2001; Elanchmezhian & Mandal, 2011) [23, 11]. The main objectives of this study are: (i) to provide alternate methods to propagate, cultivate and conserve the important medicinal wild Greek Sage (*Salvia fruticosa* Mill.) from Al-Jabal Al-Akhdar area (Balagrae, Al-Bayda, Libya) using tissue culture technology. (ii) to develop an efficient and reproducible, regeneration protocol for *S. fruticosa* via indirect organogenesis using different concentrations of growth regulators and different explants.(iii) to determine the proper environment for callus production and micropropagation of this invaluable medicinal plant.

Materials and Methods

This study was carried out in the Central Laboratory, Omar AL-Mukhtar University and at Agricultural Research Center, Libya during the period from 2019 to 2021.

Study site: Balagrae (about 10 km south of Al-Baida, 32° 70– 32° 77 N 21° 70 – 21° 68 E, 522.5 m height above sea level) was chosen as the study site because it is the most diversified woodland close to Omar Al-Mukhtar University campus.

Plant material: The plant materials (seeds, leaves, stems and roots) of *S. fruticosa* (healthy and mature) used in this study were collected from the mother plant shrubs growing in Balagrae area, in August 2019. The plant was identified and authenticated through the Silphium Herbarium in Botany Department, Science Faculty, Omar Al-Mukhtar University, Al-Baida – Libya.

Basal medium preparation: As shown in Table 1, full Murashige & Skoog medium (MS, 1962) (4.32 mg L^{-1}) nutrient salt medium and ascorbic acid (AS) (10 mg L^{-1}) was used for callus and organogenesis (shooting and rooting) media. Different plant growth regulators where used [2,4-D, NAA and BAP], the medium's pH was adjusted to 5.8 with 1N NaOH or 1N HCl, then solidified with 6 g L^{-1} agar before autoclaving at 121ºC and 1.2 kg cm^{-2} for 20 minutes. 30 ml of autoclaved medium was poured into each sterile glass culture vial (250 ml jar) for *in vitro* callus and indirect organogenesis media. The jars were stored at room temperature for at least 3 days before use under complete darkness to examine contamination.

Table 1: MS medium (Murashige and Skoog 1962) composition.

Ingredients	Amount (mg/L)
Macronutrients	
NH_4NO_3	1650.00
KNO_3	1900.00
$CaCl_2.2H_2O$	440.00

$MgSO_4.7H_2O$	370.00
KH_2PO_4	170.00
Micronutrients	
KI	0.83
H_3BO_3	6.20
$MnSO_4.4H_2O$	22.30
$ZnSO_4.7H_2O$	8.60
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2$	0.025
Iron stock	
$FeSO_4.7H_2O$	27.80
$Na_2.EDTA.2H_2O$	37.30
Vitamins	
Myo-inositol	100.00
Nicotinic acid	1.00
Pyridoxine HCl	1.00
Thiamine HCl	10.00
Glycine	2.00
Sucrose (g)	30.00
Agar (g)	8.00

Callus Induction
Explants preparation

Original field-grown *S. fruticosa* plants (2-year-old) were used as the explant source. The explants (leaf, stem and root) were cut into convenient sizes using a scalpel blade, aseptically 3-4 cm in length from the stem, root, and 4 mm^2 sizes from the leaf. The sterilization steps were identical to the sterilization methods followed for seeds. In complete aseptic conditions sterilized explants were cultured on different callus induction media (CIM) consisting of full MS basal medium and modified as follows:

CIM0= control (MS free growth regulators)
CIM1= MS + 0.5 mg L^{-1} 2, 4-D
CIM2= MS + 0.5 mg L^{-1} NAA
CIM3= MS + 0.5 mg L^{-1} BA
CIM4= MS + 0.5 mg L^{-1} 2, 4-D + 0.5 mg L^{-1} NAA
CIM5= MS + 0.5 mg L^{-1} 2, 4-D + 0.5 mg L^{-1} BA
CIM6= MS + 0.5 mg L^{-1} NAA + 0.5 mg L^{-1} BA

Three explants were inoculated per jar and twelve jar cultures per each treatment were utilized. All cultures were incubated in a controlled growth chamber, at 26 ±1°C under normal conditions. All cultures were sub-cultured on the same fresh callus induction media after 3 weeks from incubation while non-responded treatments were excluded. Callus frequency (%) and certain growth traits (fresh, dry weights in mg/jar) and water content (%) were observed and calculated after five weeks from culturing. Callus characters (type and color) were recorded and callus growth score was assessed by visual rating into four categories: prolific (4), good (3), medium (2) and poor (1).

Callus frequency

The frequency of developing calli was calculated according to Sah *et al*., (2014) as follows:

$$\text{Calli (\%)} = \frac{\text{Number of explants exhibiting calli formation}}{\text{Number of explants cultured}} \times 100$$

Growth parameters and water content analysis
Calli fresh weight (mg/jar)

Callus fresh weight was taken for all treatments after five weeks (mg/jar) from cultures.

Calli dry weight (mg/jar)

The calli were dried in a hot-air oven at 60 °C for a few days until the dry weight was stabilized (mg/jar).
Water content percentage was recorded following Lai and Liu (1988) method in which: (fresh weight - dry weight / fresh weight) × 100 %.

Statistical analysis

The test of least significant using difference (L.S.D) at the level of 0.05% significance was used to examine differences among treatment means and interactions. Data were statistically analyzed using the MSTAT-C software package according to the method described by Freed *et al.,* (1989).

Results

Callus initiation

Calli induction was observed within 14-21 days of culturing leaf, stem and root explants of *S. fruticosa* on MS medium supplemented with 2,4- D only (Figure 1 a-c) as well as in a medium received 2,4- D in combinations with BA. However, no callus formation was observed as a result of culturing on the other used media (Table 2). Exposure to 0.5 mg L^{-1} 2, 4-D only (CIM_1) gave the best result of callus formation, which was initiated after two weeks of cultivation. Several types of calli cultures were distinguishable based on their appearance (Table 2), cultured leaf explants on CIM_1 medium produced generally light greenish calli (Figure 1 a), while stem explants gave rise to brownish-white, nodular, friable callus (Figure 1 b) and induce callus brownish nodular and friable with root after three weeks (Figure 1 c), (Table 2; Figure 1 a-c).

While Exposure to 0.5 mg L^{-1} 2, 4-D + 0.5 mg L^{-1} BAP (CIM5) gave callus formation which was initiated after 5 weeks of cultivation Several types of calli cultures were distinguishable based on their appearance (Table 2), cultured leaf explants on CIM5 medium produced generally light green (Figure 2 a), while stem explants gave rise to brownish, nodular, friable callus (Figure 2 b) and induce callus brownish nodular and friable with root after three weeks (Figure 2 c), (Table 2; Figure 2 a-c).

Interaction between media composition and types of explants (leaf, stem and root) on frequency, fresh/dry weights and water content under normal cultivation conditions, explored those leaf explants responded with high significant levels (70%, 2.841, 0.186 g/jar and 60%) respectively then root (62%, 1.321, 0.034 and 33%) and last, stem (22%, 0.123, 0.031 and 16%) when cultured on CIM_5 medium (Table 3).

Adding 0.5 mg L^{-1} BA only (CIM_3), gave rise to directly adventitious shoots from stem explants, while leaf and roots were unable (Table 2; Figure 3). Combined of 0.5 mg L^{-1} 2, 4-D with 0.5 mg L^{-1} NAA (CIM_4) gave rise to directly adventitious shoots and roots (plantlets) from root explants one month after cultivation (Table 2; Figure 4)

Table 2: Effect of MS media composition on various morphological responses and callus character of different explants derived from *in vitro* germinated of *S. fruticosa* seedlings. Data were recorded after five weeks of culture under normal conditions.

Morphological response and characterization of callus				
Explants type Media composition	**Leaf**	**Stem**	**Root**	**Callus initiation (day)**
CIM_0	No response	No response	No response	-
CIM_1	Callus^{++} Light Green - Nodular and compact	Callus $^{++}$Brownish white - Nodular and Friable	Callus $^{++}$Brownish - Nodular and Friable	14^a
CIM_2	No response	No response	No response	-
CIM_3	No response	No response callus- Adventitious shoot	No response	-
CIM_4	No response	No response	No response callus- Plantlets regenerated	-
CIM_5	Callus^{+++} Light Green- Nodular and Friable	Callus^{+} - Brownish - Nodular and Friable	Callus^{+} - Brownish - Nodular and Friable	21^b
CIM_6	No response	No response	No response	-

Where: + means low; ++ Moderate and +++ High callusing.

Means having the same letters in a column were not significantly different at $p<0.05$

Where:-

CIM_0= control (MS free growth regulators)

CIM_1= MS + 0.5 mg L^{-1} 2, 4-D

CIM_2= MS + 0.5 mg L^{-1} NAA

C IM_3= MS + 0.5 mg L^{-1} BA

CIM_4= MS + 0.5 mg L^{-1} 2, 4-D + 0.5 mg L^{-1} NAA

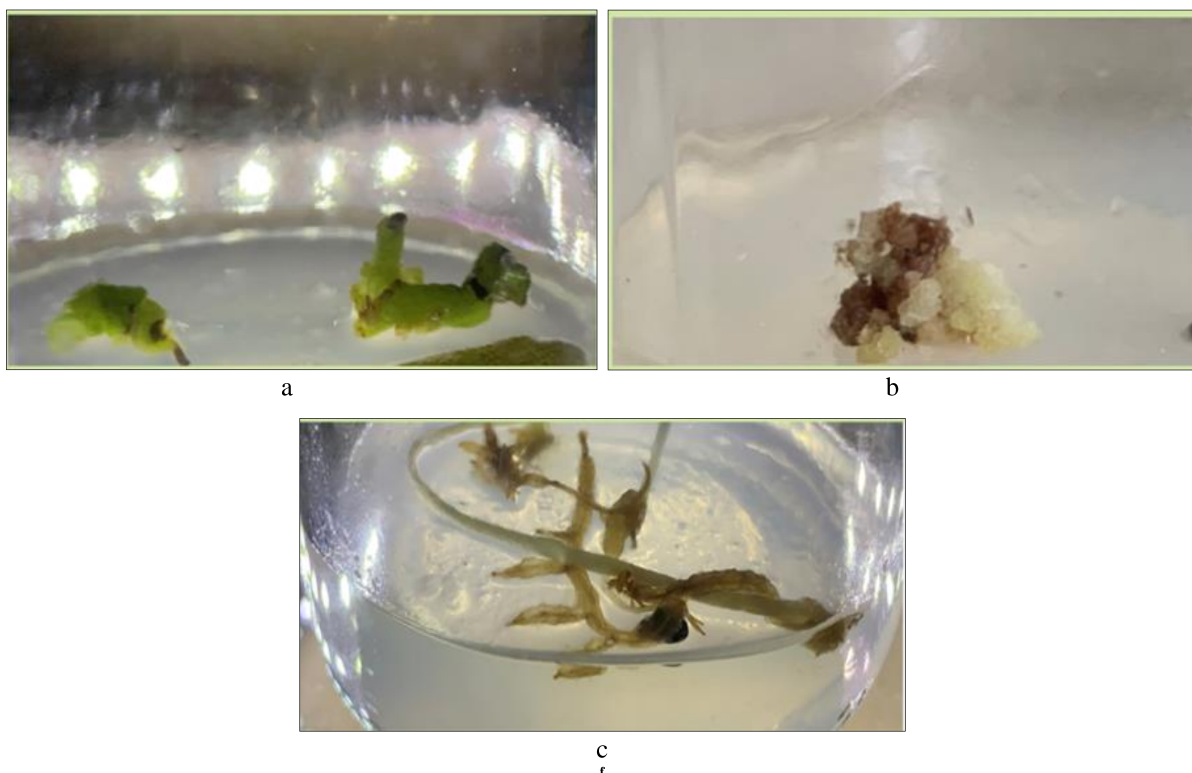
CIM_5= MS + 0.5 mg L^{-1} 2, 4-D + 0.5 mg L^{-1} BA

CIM_6= MS + 0.5 mg L^{-1} NAA + 0.5 mg L^{-1} BA

Table 3: Effect of interaction between explant types and media composition on the frequency of callus; calli fresh and dry weights and water content of different explants derived from *in vitro* germinated of *S. fruticosa* seedlings. Data were recorded after five weeks of culture under normal conditions. Media CIM_0 and CIM_2- CIM_4 and CIM_6 using different explants failed to induce callus and were excluded.

Explant Media Composition	Callus growth parameters											
	Callus formation (%)			Fresh weight (mgjar)			Dry weight (mg/jar)			Water content (%)		
	Leaf	Stem	Root	Leaf	Stem	Root	leaf	stem	Root	leaf	Stem	Root
CIM_1	70^a	22^d	62^b	2.841^a	0.123^e	1.321^b	0.186^a	0.031^c	0.034^c	60^a	16^f	33^e
CIM_5	62^b	15^e	42^c	0.821^c	0.107^f	0.422^d	0.030^c	0.010^d	0.062^b	54^b	24^d	46^c

Means having the same letters in a column were not significantly different at $p<0.05$
Where:-
CIM_1= MS + 0.5 mg L^{-1} 2, 4-D
CIM_5= MS + 0.5 mg L^{-1} 2, 4-D + 0.5 m



a b

c

Fig 1: Different types of calli derived from leaf (a), stem (b) and root (c) explants of *S. fruticosa* after two weeks of culturing on MS medium containing 0.5 mg L^{-1} 2, 4-D (CIM_1) under normal conditions.

a **b**

c

Fig 2: Different types of calli derived from leaf (a), stem (b) and root (c) explants of *S. fruticosa* after five weeks of culturing on MS medium containing 0.5 mg L^{-1} 2, 4-D + 0.5 mg L^{-1} BAP (CIM_5) under normal conditions.

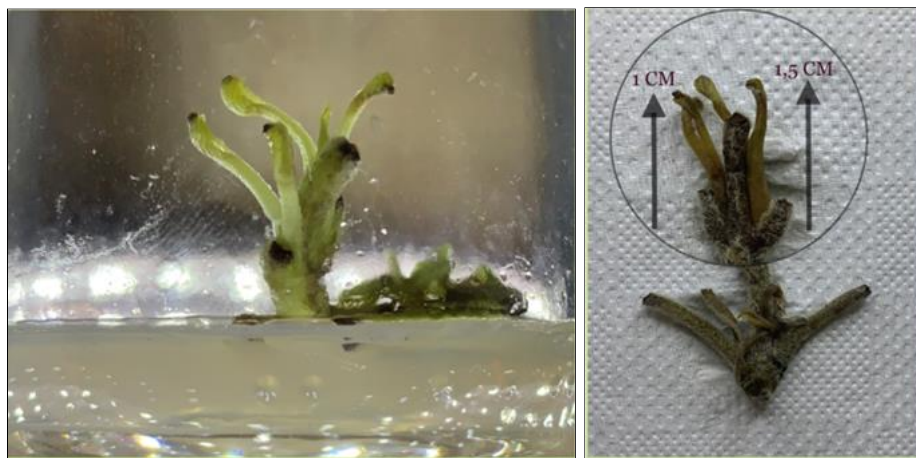


Fig 3: Adventitious shoot induction indirect of stem explants *of S. fruticosa* after one month of culturing on MS medium containing 0.5 mg L^{-1} BA (CIM_3) and cultures were incubated under normal conditions.

Fig 4: Plantlets regenerated from root explants after four weeks of inoculating on MS medium containing 0.5 mg L^{-1} 2, 4-D+ MS + 0.5 mg L^{-1} NAA (CIM_4).

Discussion

Plant tissue culture is a work of art and science, which enhances genetic diversity, creates virus-free plants and expands micropropagation under hygienic situations in the short period (Birch, 1997). Plant cells have high flexible prospective for cell differentiation (Neely, 1979). The pivotal phase in the beginning of plants into tissue culture is to get cultures free from microbial contamination.

In this current study, the developed effective and reproducible system for micropropagation confirmed production of numerous *S. fruticosa* plants, which could be used as a promising foundation of secondary metabolites. This should facilitate the pressure and reduce over-collection of this species from the wild. That makes this technique suitable for ex situ conservation and for commercial production of *S. fruticosa* plantlets.

The formation of an efficient protocol for callus induction is tremendously vital as a first stage towards many molecular drives. It was found that growth regulator type and concentration had a substantial influence on the callus induction day, frequency, callus growth score, and certain growth traits (fresh, dry weight and water content).

Maximum callus induction was obtained for explants when were cultured on MS medium enriched with 2, 4-D (0.5 mg L^{-1}), which was initiated after two weeks of cultivation. Among all the growth regulators used, 2,4-D was found to be the most effective growth regulator for *S. fruticosa* callus induction.

This result is in accordance with previous research (Bennett *et al*., 1998, Mansur *et al*., 2018). They confirmed that auxins 2,4-D symbolize one of the most significant classes of signaling molecules involved in the regulation of cell division, cell elongation and cell differentiation in higher plants.

The highest scored factors of callus (day-14, 90 %, good, 2.841, 0.186 mg fresh/dry weight, and 60 % water content) were obtained from culturing leaf explants of *S. fruticosa* on MS medium supplemented with 2,4- D only compared to other media composition. For this reason 2,4-D not only mimics auxins (concentration-dependent mode of action) (Raghavan *et al.,* 2006) [32] but also, may preferentially induce stress responses in plant cells. Earlier researches showed that the existence of synthetic auxin, 2,4-D was a significant force factor for effective *Oryza sativa* L. callus induction (Joyia & Khan, 2013) [18].

However, because each cultivar has specific features determined by its genotype, specific concentrations of growth regulators are required.

Hiei & Komari, (2008) [17] discovered that in case of callus induction of rice, 2, 4-D alone or in mixture with 1-naphthalene acetic acid (NAA) have been effectively induced (callus derived) from seed.

TrejoTapia *et al.* (2002) proposed that sole auxin was a better substitute rather than using the combination of auxin (NAA, BA and 2, 4 -D). AL-Hussaini *et al. (*2015) [4] moreover tried diverse mixtures of hormones (2 mg $^{l-}$BA +2.5 mg l^{-1}NAA; 2 mg $^{l-}$1BA+ 2 mg -12,4-D; 2 mg $^{l-}$12,4-D) beside free hormone medium as mechanism to improve an active protocol for most favorable callus induction and plant regeneration in 4 potato variations among them 2 mg l^{-1} BA + 2.0 mg $^{l-}$2,4-D and 2 mg l^{-1}2,4-D alone provided good reaction and a good callus propagation. In the meantime the same hormonal composition is not appropriate for all rice variations; the adjusting media were differentiated to affect the genotypic influence for certain rice variations.

To our best knowledge, for the first time this study established a plant regeneration system to reproduce Greek sage plants via callus induction and indirect organogenesis. The frequency of micropropagation in *S. fruticosa* is chiefly influenced by concentration of plant growth regulators, and the source of the explant tissues. This finding is preparatory, additional research of the impacts of genotype, pretreatment and culture condition on the propagation and regeneration at each of the developmental stages is required to enhance the effectiveness of plant regeneration in Greek sage.

Based on literature review, it was stated that suitable concentration of hormone for callus induction in explants and seedling reproduction from callus will be diverse dependent on plant species, hormone type utilized in culture environment, growth stage of maternal plant and explant type. Therefore, in accordance with anticipated goal in tissue culture procedures and type of used explant, proper hormonal treatment should be applied. Also, researchers proved that the capability of tissue culture and plant regeneration from callus is linked to heredity and many genes in the nucleus and cytoplasm could regulate it (Lemraski *et al*., 2014) [21].

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