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Influence of Gibberellic Acid (GA3) and kinetin (Kin) on *in vitro* Regeneration of *Salvia fruiticosa*

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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 work is to establish an effective protocol for producing *S, fruiticosa* in vitro, which would contribute to resolving the state of secondary dormancy and conservation this species. Effect of auxin and Cytokinins on seed germination percentage, morphological plant characters (plant length and seed vigor index) and photosynthetic pigments (Chlorophyll a and b) of seeds cultured on half-salt strength MS medium supplemented with 0.05 mg L^{-1} Gibberellic acid (GA_3) and 0.05 mg L^{-1} kinetin (Kin). GA_3 showed significant increase of germination percentages (55%) compared to Kin (20%). Highest seed vigor index was achieved with kin (53.93) compared to (12.55) with GA_3. There were no statistically significant differences differences in the plant length between different treatments. Chlorophyll (a) and (b) in leaves were high significantly increased with Kin compared to GA_3.

تأثير حمض الجبريليك (GA3) والكينيتين (Kin) على تجديد *Salvia fruiticosal* **في المختبر**

رابحة منصور هبة فحيمة صباح لملوم

تم انشاء بروتوكول لأجل زراعة لتفاح الشاهي في المختبر (Salvia fruticosa)، وهو نوع مهدد بالانقراض بسبب أهميته كنبات طبي في منطقة الجبل الأخضر، ليبيا. كان الهدف من هذه الدراسة هو تطوير طريقة فعالة للتكاثر الدقيق لـ S. fruticosa ، والتي من شأنها أن تساهم في الحفاظ على هذا النوع وانتشاره تجاريًا. تأثير منظمات نمو النبات المختلفة على معدل إنبات البذور والصفات المورفولوجية للنبات (ارتفاع النبات ومؤشر قوة البذور) وأصباغ التمثيل الضوئي (الكلوروفيل أ و ب) للبذور المزروعة على وسط ملحي موراشيج وسكوج (MS) بنصف القوة المضاف إلى 0.05 ملغم حمض الجبريليك (GA3) .L-1 و 0.05 ملغم L-1 كينيتين (Kin).أظهر GA3 زيادة معنوية في معدلات الإنبات (55%) مقارنة بـ (20%) Kin . تم تحقيق أعلى مؤشر قوة للبذور باستخدام (53.93) Kin مقارنة بـ (12.55) مع GA3 لم تكن هناك فروق معنوية في طول النبات بين المعاملات المختلفة. كان الكلوروفيل أ و ب في الأوراق أعلى بكثير مع معاملة الكينيتين.

INTRODUCTION

Salvia fruticosa is aromatic shrubby plant that grows in the highlands of the Al-Jabal Al-Akhdar area. It is one of the largest species in the Lamiaceae family .It is considered one of the medicinal plants that has many useful advantages, as it is rich in aromatic oils as well as important medicinal compounds (Carmona et al., 2005 ; Giweli *et al*., 2013 and Cvetkovikj *et al*., 2015). For this reason, we chose the fruticosa plant in this study because it is widely used in traditional treatment and also it is threatened with extinction and lacks a lot of

information.

In spite of the fact that Salvia, one of the most important perennial medicinal shrubs that has been used in traditional medicine practices since prehistoric times, it does not last for more than three or four years without deteriorating, so plantings must be repeated at least every four years. Although impressive advance has been made within the field of the in vitro generation of different secondary metabolites, such as rosmarinic acid and cryptotanshinone, the application of biotechnological strategies for the propagation of these species is constrained. This could be due to the fact that most Salvia species can be propagated simply by routinely excising shoots from three-year-old parent plants (Pierozan *et al*,.2009). Reproduction of *S. fruticosa*, by seed, is a process restricted by a rather low seed germination rate. Biotechnology techniques are considered one of the most important means of preserving rare plants and those threatened by deterioration or extinction. As a result of the over-harvest of *S. fruticosa*, finding ways to preserve this plant species via *in vitro* propagation has become necessary.

At present, interest has been paid to in vitro plant tissue culture technology because it provides radical solutions to address problems associated with plant growth, including breaking primary and secondary dormancy, in addition to providing the ability to assist in the selection of desired plant varieties.

The purpose of this study was to develop an effective protocol for seed germination and seedling growth. *fruticosa in vitro.*

Materials and Methods

Basal medium preparation:

For *in vitro* seedlings germination, half-salt strength medium (2.16 mg L^{-1}) Murashige and Skoog (MS, 1962) augmented with 30 g L^{-1} sucrose, 0.05 mg L^{-1}, Gibberellic acid (GA_3) and 0.05 mg L^{-1} kinetin (Kin) was used (Lee *et al*., 2002).

To investigate how Gibberellic acid enhances *S. fruticosa* breaking dormancy and germination when applied to germination induction medium (GIM) as compared to kinetin. Mature seeds of *S. fruticosa* were washed with running tap water and soap for 5 min. For surface sterilization of *S. fruticosa* seeds, ethanol and Clorox (sodium hypochlorite, NaOCl) were applied. *Salvia fruticosa* seeds were taken to the laminar air flow cabinet in which they surface sterilized by placing in 70% ethanol for 1 min and were immediately rinsed with double distilled water to remove ethanol traces.

Then the seeds were rinsed and shaken in sodium hypochlorite (NaOCl 10 %) for 20 min. The seeds were washed thoroughly 5-6 times with double distilled water to remove NaOCl according to Garcia *et al.* (1999) method. This step aimed to remove microorganisms from the surface of the seeds; bacteria and fungus are the major contaminants *in vitro* culture. An equal number (four) of sterilized seeds were inoculated in jars; twelve jars per each treatment were utilized. After 4 inoculations, forceps were kept in ethanol and then were burned with a flame to achieve maximum sterilization and the inoculation was carried out close to the flame to minimize the risk of contamination. Based on multiple treatments, seeds were planted in three groups: the first group was planted on MS without growth regulators (GIM_0 control) while the second was cultured on MS supplemented with0.05mg L^{-1} of GA_3 ($GIM_{1)}$ and the third group of seeds were planted on MS with 0.05 mg L^{-1} of Kin (GIM_2). The culture jars were incubated in dark conditions at 24-25℃. At the end of this step, germination percentages were calculated twelve days after culturing according to the equation of Mishra *et al.* (2021) and after twenty-five days vigor index according to Abdul-Baki and Anderson, (1973) and photosynthetic pigment contents
(Arnon 1949) were calculated.

Results and Discussion

In the present study, no germination induction was found in basal free hormone media (GIM_0). Germination in the present study means the emergence of both root and shoot as shown in Figure 1. In this study, components of medium significantly influenced germination and related traits of *S. fruticosa*. MS with 0.05 mg L^{-1} GA_3 (GIM_1) showed significant increment (effective treatment(for germination percentage (55%) compared to MS medium supplemented with 0.05 mg L^{-1} Kin)GIM_2) Table 1. Seed germination on MS medium supplemented with GA_3 was induced within 7 days of culture. Whereas, seed germination was differentiated on MS supplemented with Kin but showed a longer period (10 days) in culture. According to the preliminary experiment on seed germination, it was found that GIM_1 was found to be the best medium for seeds germination percentage but limited growth and development even if they were maintained compared to GIM_2.

Morphological plant characters:

Data in Table 2 represent the applied different plant growth regulators' effects on plant length (cm) and seed vigor index of *S. fruticosa* seedlings. Among the plant growth hormones, a statistically significant decrease was observed for seed vigor index value when GA_3 was used. i.e. GA_3 has the lowest effect. In comparison, the highest seed vigor index was achieved with MS medium supplemented with 0.05 mg L-1 kin (GIM_2) which was 53.93 compared to 12.55 for MS medium supplemented with 0.05 mg L^{-1} GA_3 (GIM_1). Experiments showed that there were no significant differences in the plant height (cm) within different treatments (Figure 2), although there were significant differences in seed vigor index.

Photosynthetic pigments content (Chlorophyll *a* and *b* mg g^{-1} FW) in leaves of *S. fruticosa* seedling were high significantly increased with 0.05 mg L^{-1} Kin) GIM_2) compared to GIM_1 (Table 2). Chlorophyll *a* content was higher over the chlorophyll *b* in two of the treatment of *S. fruticosa* leaves. The results showed that chlorophyll a was higher (0.650 mg g^{-1} FW) in 0.05 mg L^{-1} Kin, however, concentrations of 0.05 mg L^{-1} GA_3 recorded low Chlorophyll *a* (0.310 mg g^{-1} FW) content (Table 3). The present data illustrated in Table 3 showed that MS medium supplemented with 0.05 mg L^{-1} GA_3 (GIM_1) recorded low Chlorophyll *b* content (0.224 mg g^{-1} FW) compared to Kin treatments (Table 2).

Table (1): Seed germination of *S. fruticosa* after twelve days of culturing on half-salt strength MS medium supplemented with different plant growth regulators under normal conditions. In the present study, no seed germination was found in basal free hormone media (GIM0)

Treatment	Germination (%)
GIM_0	0^c
GIM_1	55^a
GIM_2	20^b

Values with the same letters in the column were not significantly different p<0.

Table (2): Morphological plant characters (plant length and seed vigor index) and photosynthetic pigments (Chlorophyll *a* and *b*) of *S. fruticosa* (data were recorded after 20 days cultured on half-salt strength MS medium under normal conditions).

Parameter / Treatment	Morphological plant characters		Photosynthetic pigments (mg g^{-1} FW)	
	Plant length (cm)	**Vigor index**	**Chlorophyll *a***	**Chlorophyll *b***
GIM_1	13.55^a	12.55^b	0.310^b	0.224^d
GIM_2	11.00^a	53.93^a	0.650^a	0.475^a

Values with the same letters in the column were not significantly different p<0.05

Figure (1): Germination of *S. fruticosa* mature embryos cultivated on MS modified with GA_3.

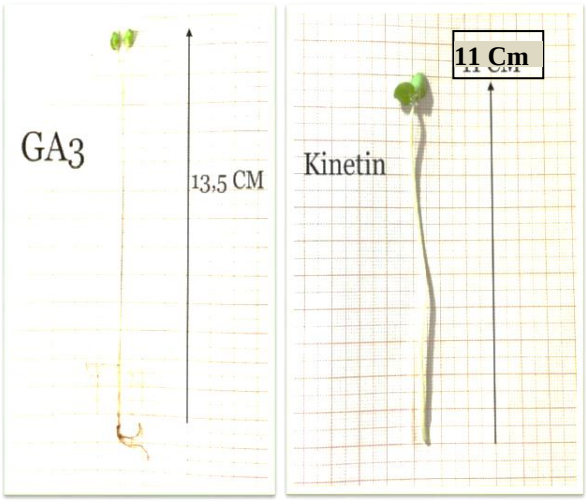

Figure (2): *In vitro S. fruticosa* seed germination on half-salt strength of MS medium with GA_3 and Kin growth regulators After 25 days of culture under normal conditions.

In this present study, the developed efficient and reproducible micropropagation system confirmed the production of several *Salvia fruticosa* plants, which can be used as a promising basis for secondary

metabolites. This would reduce the over-harvesting of this plant from nature. Therefore, this technique is considered suitable for plant conservation outside the natural habitat and for the commercial production of *Salvia fruticosa* plants .The developmental path proceeding to break dormancy and germination is true establishment of totipotency of plant cells. Throughout this practice, plant cells, under suitable circumstances, divide and differentiate into seedling. This viewed developmental path was adopted for decoding the elaborate molecular regulatory mechanisms of the extracellular kinetin (KIN) and Gibberellic acid (GA_3) application result to reveal its signaling path expressions (gene expression patters) during this process signaling applying *Salvia fruticosa*.

The current work aims to examine the hormonal role during breaking dormancy and germination. The hormones under investigation (KIN and GA_3) at concentration of 0.05 mg L^{-1} were applied. The present findings recommended that GA_3 have a vital role when compared with KIN during *S. fruticosa* seed germination's process.

Consequently, balance in the level of several hormones with each other, which we call the multi-hormone system, where treatment from the outside with a particular hormone affects the relationship of the internal hormones with each other, whether stimulating or inhibitory, and thus the result of this is the lack of the resulting effect in a physiological picture that is determined based on the levels of these hormones internally and their relationship with each other (Pitarokil *et al.,* 2003 and Elancmezhian *et al.,* 2011). Therefore, making modifications to the nutrient medium, with components that may mimic the inductive condition (providing a deficiency or counteracting a surplus), to develop or improve laboratory procedures for embryo production and transformation to seedling (Lee *et al.,* 2002 and El-Wahab *et al.,* 2015).

The use of exogenous GA3 promotes pivotal functions in different stages of plant biology, which contain cell division and elongation, root formation, differentiation of vascular bundles (xylem and phloem), breaking seed dormancy, seed germination, improving the immune system, inflorescence formation and reproduction (Wei & Li, 2016).

This study is considered the first of its kind to produce *S. fruticosa* in vitro by indirectly stimulating the process of callus and organ formation. Microproliferation in *S. fruticosa* is mainly affected by the level of growth regulator; the type and age of the transplanted explant. This result is preliminary, and several experiments and research into the effects of genotype and culture condition on reproduction and regeneration at each growth stage are needed to enhance the effectiveness of plant regeneration in *S. fruticosa.*. According to previous studies, it was reported that the appropriate level of hormone for callus formation on explant grown in vitro and the emergence of seedlings on callus will be diverse depending on the types and age of plant part, the type of hormone used in the culture, the physiological condition of the mother plant (Al Sheef *et al.,* 2013) . Therefore, according to the expected goal of the in vitro propagation procedure and the explant types, the appropriate auxin treatment must be chosen. Researchers have also shown that biotechnology and the ability of a plant cell to divide and form a plant is linked to gene expression, which can be regulated by many genes in the nucleus (Kintzios, 2000 and Lemraski *et al.,* 2014).

CONCLUSION

Prevailing knowledge with tissue culture of Salvia sp. is restricted to a small number of species and chiefly focuses on callus induction from numerous explants so that to ease the in vitro production of secondary metabolites. The techniques of plant tissue culture would be usful for the safety of endangered or rare species. *Salvia fruticosa* is a medicinal plant and an efficient in vitro conservation system was established in this current study. The examined plants of *S. fruticosa* reacted well in tissue culture (under the conditions defined) and revealed an exceptional potential for direct organogenesis. The in vitro response varied depending on the explant and the growth regulator concentrations used. This research established valid protocols for efficient propagation of *S. fruticosa*. In summary, the micropropagation protocol described in this present study provides rapid and large scale production of plantlets of *S. friuticosa* Mill species probable, beginning with a small amount of plant material.

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