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*Full Length Research Paper*

# Genomic DNA extraction from seed induced callus and explants in *Salvia* L. species for utilization in secondary metabolite production

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***Salvia*, the largest genus of Lamiaceae, includes about 900 species, widespread throughout the world. This genus is represented, in Turkish flora, by 88 species and 93 taxa, 45 of which are endemic. Some members of this genus are of economic importance since they have been used as flavoring agents in perfumery and cosmetics. Despite the medicinal potential of plants in Turkey being considerable, knowledge of this area and studies on these crops is scarce. Including *Salvia* L. species, in some medicinal plants low callus production, especially leave-based ones, is the main problem in *in vitro* culture, due to phenolic compounds. Callus induction, therefore, from plant's non-specialized tissues, for example, seeds, is inevitable. The six species of *Salvia* L. growing naturally in Anatolia (*Salvia aetopis* L., *Salvia cadmica Boiss.*, *Salvia candidissima* Vahl *ssp. occidentalis* Hedge, *Salvia cryptantha* Montbret et Aucher ex Bentham (endemic to Turkey), *Salvia tomentosa* Miller and *Salvia verticillata* L. *ssp. verticillata* L.) were selected. Callus of the seeds of the aforementioned species was initiated on MS basal media supplemented with combinations of indole-butyric acid (IBA) and picloram. Varied mass callus induction was observed in all media but, *Salvia tomentosa* Miller was found to be most efficient species in callusing. Moreover, applying the Lefort method, the extracted genomic DNA for all the samples was pure and ideal.**

**Key words:** Callus, genomic DNA extraction, *in vitro* culture, *Salvia* L. species

## INTRODUCTION

Many plant species from genus *Salvia* have long been recognized as medicinal herbs in the traditional art of healing, and their derivatives continue to be important components of contemporary phytopharmaceuticals. The rich assortment of secondary metabolites in plants from genus *Salvia* has created a considerable interest in the research community for their production in cell or tissue culture (Botla et al., 2000). An unusually large number of useful secondary metabolites, belonging to various chemical groups, such as essential oils, terpenoid compounds and phenolic derivatives, have been isolated from the genus, which features prominently in the pharmacopoeias of many countries throughout the world (Kintzios et al., 1999).

Despite the importance of the aforementioned species within the Labiatae family, the application of biotechnological methods for the propagation of this species to recover high valuable metabolites appears rather limited (Avato et al., 2005). On the other hand, in *Salvia* species, low callus production is the main problem in *in vitro* culture of seeds (Rani and Grover, 1999). The increasing demand for useful secondary metabolites has intensified the application of biotechnological methods to reproduce high yielding plants under controlled growing conditions and/or to obtain homogenous and stable genotypes. A growing interest in the development of efficient protocols to micropropagate certain species of *Salvia* has also been evident in the more recent years, sometimes correlated with the production of higher triterpenes and phenolic constituents (Kintzios, 2000; Hippolyte et al., 1992; Santos-Gomes et al., 2002; Arikat et al., 2004; Cuenca and Amo-Marco, 2000).

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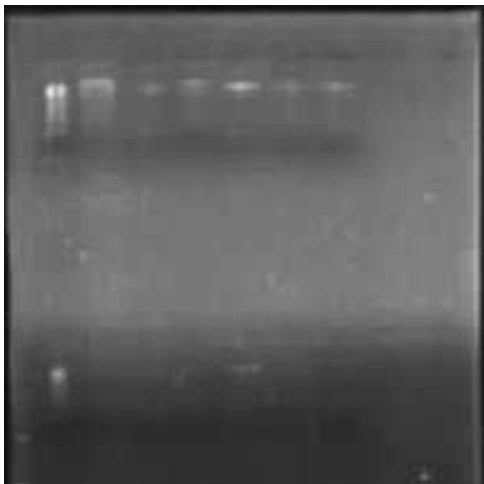

**Figure 1.** Agarose gel electrophoresis of typical enzymatically isolated DNA from *Salvia* L. species in the following order (respectively from left to right): *S. tomentosa, S. verticillata ssp. verticillata*, *S. cryptantha*, S*. cadmica, S. candidissima ssp. occidentalis* and *S. aetopis.*

Here, growth regulator concentrations in culture medium are critical for control of growth and morphogenesis. Generally, high concentration of auxins and low cytokinins in the medium promote abundant cell proliferation with the formation of callus. In the present study, verifying a preliminary approach, describes the establishment of callus and DNA extraction of some *Salvia* species, which could be extended to the other crops having the same inconvenience.

## MATERIALS AND METHODS

### Plant material

The intact seeds of *salvia* species (including *Salvia aetopis* L., *Salvia cadmica* Boiss., *Salvia candidissima* Vahl ssp. *occidentalis* Hedge, *Salvia cryptantha* Montbret et Aucher ex Bentham, *Salvia tomentosa* Miller and *Salvia verticillata* L. ssp. *verticillata* L.) were surface-sterilized with 0.1% (w\v) mercuric chloride for 2 to 3 min, followed by rinsing in 70% (v\v) ethyl-alcohol for 30 s, then washed 3 to 4 times with double-sterlied water and inoculated on agar-solidified MS medium supplemented with different combinations of auxin derivatives including IBA and picloram.

### Establishment of callus culture media

Basal medium used for initial set of experiment for proliferation consisted of MS salt, sucrose and agar. As for *in vitro* cultures, the basic culture medium (BM) was used, containing the macronutrients according to Murashige and Skoog (1962), sucrose (40 g $L^{-1}$) and agar (7 g $L^{-1}$). The pH of the medium was adjusted to 5.6 to 5.8. Sterilization of culture media was performed in autoclave at 121°C for 20 min; the culture of explants was instead carried out under a horizontal laminar flow hood to ensure the necessary sterile conditions. Matereials were kept in the darkness at 24°C. After 6 weeks of culture, the efficacy of each medium variant was determined by observing the percentage of primary explants developing callus, shoots and the rate of vitrification, browning and contamination (Avato et al., 2005).

### DNA extraction

The seed-induced callus was detached from the medium and used for DNA isolation.

### Reagents and solutions

The chemicals used in the isolation of DNA for 50 ml were:

1) 4 ml EDTA (50 mM, pH 8.0)
2) 10 ml LiCl (4M)
3) 1 g CTAP (1%)
4) 2 g PVP (2%)
5) 0.5 ml TWEEN 20 (0.5%)
6) 10 µl 2-Merkaptoethanol
7) Rnase-A (Sigma R9009); 10 mg/ml

### DNA Isolation method

DNA was extracted using the protocol described by Lefort et al. (1998). At the end the quality and concentration of the DNA on a 1% agarose gel was checked (Figure 1).

### Agarose gel electrophoresis

Genomic DNA products were electrophoresed on 1% agarose gel using 0.1x TAE buffer (10 mM Tris HCl and 1 mM EDTA pH. 8.0) and visualized by ethidium bromide staining. The patterns were photographed and stored as digital pictures in gel documentation system. The DNA samples of every plants were confirmed by repeating each experiment four times.

## RESULTS AND DISCUSSION

### Callus culture growth characteristics

Callogenesis had serious difficulties in the stabilization phase. The percentage of plantlets suffering from browning, vitrification and contamination was high especially for *S. candidissima* and *S. verticillata.* Medium supplemented with IBA regulator produced no positive results, getting the percentage of crops suffering from vitrification and browning even higher. It was the case for two concentration applied (5 and 10 mg $L^{-1}$) (Table 1). Many explants developed brown necrotic areas, which in some cases led to explant decline and death. Induction of callus was almost completely inhibited in the absence of picloram in the medium indicating the importance of exogenous growth regulators for inducing cell division and consequently callus formation. Further, this drastic decline in callogenesis could be related to the adverse effect of highly IBA nutritional applied (10 mg $L^{-1}$), nevertheless, it was true for small amounts used (5 mg $L^{-1}$) in the study.

**Table 1.** Callus induction in seed explants of *Salvia* species inoculated on MS supplemented with different concentration of IBA and picloram.

| *Salvia* species | IBA | | Picloram | Period | Remarks |
|---|---|---|---|---|---|
| | 10 mg $L^{-1}$ | 5 mg $L^{-1}$ | (4 mg $L^{-1}$) | | |
| *S. aetopis* | - | + | ++ | 6 | Greenish callus and hard in texture with axillary shoots |
| *S. cadmica* | + | + | +++ | 6 | White loose callus |
| *S. candidissima* | - | - | + | 6 | Plae yellow callus with axillary shoot. |
| *S.cryptantha* | - | + | ++ | 6 | Light brown callus |
| *S. tomentosa* | + | ++ | ++++ | 6 | Bright-white callus |
| *S. verticillata* | - | - | + | 6 | Pale green callus with axillary shoot |

Legends: – no callus induced; + low (<0.05 g); ++ moderate (0.05 to 0.1 g), +++ high (0.1 to 0.5 g); ++++ excellent (>0.5 g).

**Table 2.** Days to visible callus induction in in vitro seed culture of *Salvia* species supplemented with picloram (4 mg $L^{-1}$) and IBA (10 and 5 mg $L^{-1}$).

| *Salvia* species | IBA | | Picloram |
|---|---|---|---|
| | 10 mg $L^{-1}$ | 5 mg $L^{-1}$ | (5 um\mol) |
| *S. aetopis* | - | $28^{a}$ | $17^{c}$ |
| *S. cadmica* | $32^{a}$ | $26^{a}$ | $14^{b}$ |
| *S. candidissima* | - | - | $19^{c}$ |
| *S. cryptantha* | $36^{b}$ | $28^{a}$ | $17^{c}$ |
| *S. tomentosa* | $31^{a}$ | $27^{a}$ | $13^{ab}$ |
| *S. verticillata* | - | - | $18^{c}$ |

Data followed by the same letters, in the column, are not significantly different.

Therefore, hormone type and concentrations play an important role in callusing process. Adequate results were only achieved with the addition of 4 mg $L^{-1}$ picloram to the growth substrate while increasing the auxin concentration above this level reduced the callusing frequency slightly. Similar results were obtained by McKently et al, (1990) and Vajranabhaiah et al, (1993). Generally, picloram treatment induced much more seeds to callogenesis than that of IBA treatment, resulted in high quality callus (Table 1).

Effeciently, callus initiation appeared, after 2 to 3 weeks, ranging from light brown, plae yellow to creamish-white and white, in color. The callus appeared at the seeds cut end in two distinct, friable and compact forms. Friable and compact callus culture sustained its initial morphological form throughout the experimental period. Table 1 gives seed-derived callus for *Salvia* L. species planted in MS media supplemented with concentrations of picloram (4 mg $L^{-1}$). Culturing response, callus color and texture showed difference according to the species. An excellent callogenesis was observed after a culture period of almost 5 weeks in *S. tomentosa* resulted in greenish-white callus following by S. *cadmica* inducing bright white callus. Whereas, the lowest frequency of callus formation was characterized in *S. verticillata* and *S. candidissima* (Table 1). It is has been shown that the content of essential oils in *Salvia* changes with the species (Kintzios, 2000; Giannouli and Kintzios, 2000). Moreover, it is known that terpenoid synthesis proceeds through a variety of intermediates and originate from different reactive carbocations (Bruneton, 1999; Croteau and Karp 1976). Dominance of one or the other compound in the essential oil of sage should be reasonably correlated with the activation of the specific metabolic pathway which may consequently affect the callogenesis process.

Days to visible callus induction were lowest in *S. verticillata* and *S. candidissima* representing 18 and 19 days, respectively, cultured in picloram supplemented medium. IBA supplemented media resulted in least callus induction percentage and had the highest number of days to visible callus, in both 10 and 5 mg $L^{-1}$ treatments (Tables 1 and 2). Here, an excellent callogenesis was observed after a culture period of almost 5 weeks in *S. tomentosa* resulted in bright-white callus. Again, genotype variability and the contribution of the compounds in the essential oil may cause significantly differences in period to visible callus.

## Shoot regeneration

Seeds planted faield to response morphogenetically to a growth regulator and no shoot generation yielded for *S.*

**Table 3.** Mean number of shoots and shoot-induced root length per callus on MS medium supplemented with picloram (4 mg $L^{-1}$) in the sixth week.

| *Salvia* species | Hypocotyle | Epicotyle | Cotyledonary node | Leaf | Root (cm) |
|---|---|---|---|---|---|
| *S. aetopis* | 17.3[ab] | 18.6[ab] | 17.8[ab] | 30.5[a] | 14.8[a] |
| *S. verticillata* | 13.2[bc] | 14.3[bc] | 13.8[bc] | 24.6[ab] | 11.3[ab] |
| *S. candidissima* | 10.6[c] | 11.2[c] | 11.7[c] | 23.7[ab] | 11.0[ab] |

Within a column, means having the same letter are not statistically significant (P = 0.01).

*cadmica, S. tomentosa and S. cryptantha*. Nevertheless, numerous globular shoots were readily formed on callus tissue of *S. aetopis, S. verticillata* and *S. candidissima* species in culture, respective of medium composition so that it could be observed in picloram supplemented medium (Table 3). Seeds cultured on 1 mg $L^{-1}$ picloram increased shoots 12 to 16 cm in length. Callus regeneration of three species are depicted in Table 3. Shoot differentiaon was observed in high frequency form leafs followed by epicotyle, cotyledonary node and hypocotyle explants. One of the strategies for ensuring regeneration from undifferentiated callus is to maintain low concentration of auxin hormons, as that of the current study. Of the three species, maximum explant regeneration was observed in *S. aetopis* follwed by *S. verticillata* and S. *candidissima* (Table 3). It seems that shoot differentiation could even be increased at the presence of optimum concentrations of cytokinins. High frequency of shoot bud differentiation was characterized on MS medium supplemented with BAP (2.5 mg $L^{-1}$) (Venkatachalam et al., 1994). Hence, the process of crop regeneration depends upon the cytokinin\auxin ratio, explants, hormones types and concentrations.

Further, in the current study the topical and bare application of picloram resulted in the initial shoots with trace callusing. Though the mechanism of this phenomenon needs to be investigated, it could be attributed to the availability of the hormone being restricted to the explant. Among the explants, the maximum number of shoots (30.5) was observed in *S. aetopis* leaves whereas the least number of shoots (10.6) was identified in the hypocotyl derived callus of *S. candidissima* (Table 3). At the end of the subculture, all of the plantlets induced white, long and slender roots on the medium contaning picloram (4 mg $L^{-1}$). Rooting percentage was observed in high frequency in *S. verticillata* followed by *S. aetopis* and *S. candidissima* (unpublished). Picloram treatment was satistically significant for roots lenght (Table 3). The root longest mean was recorded for *S. aetopis* (14.8 cm) followed by *S. verticillata* and *S. candidissima* (Table 3). This result, in agreement with that already reported fort the *S. fruticosa* (Arikat et al., 2004) and *S. sclarea* (Liu et al., 2000), supports the concept that auxin supplementation is necessary for rooting. Neither shoot regeneration nor root formation characterized in the IBA supplemented medium. However, regenerated shoots obtained from the axillary shoot base callus were rooted on MS medium containing IBA (2 and 4 mg $L^{-1}$), or IBA (2 mg $L^{-1}$) with IAA (2 mg $L^{-1}$) in the study of Rani and Grover.

## DNA extraction

Fast and reliable DNA extraction was achieved using ground, freeze-dried callus from species (Figure 1).

## Conclusion

Considering high percentage of phenolic compounds which makes the generative production hard and even impossible, establishment of techniques for regeneration *Salvia* L. species by tissue culture is inevitable. Moreover, further studies with medium components of pot and natural condition could verify the surviving species. The experiment has confirmed that callogensis as well as days to visible callus is highly sensitive to the species and the hormone-supplemented mediun employed. The investigated plants of *Salvia* species responded almost well in tissue culture and demonstrated an outstanding potential for callus production *in vitro.*

On the basis of the results obtained in the current work, we can tentatively conclude that the observed growth and production patterns of *S. tomentosa* callus cultures, in particular, offer promising perspectives, both from qualitative and quantitative point of view, for studying the relationship between genotype tested and *in vitro* secondary metabolism.

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