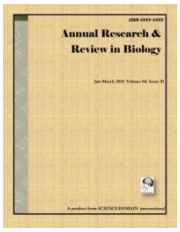

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Effect of Different Growth Regulators on Callus Induction and Plant Regeneration of *Satureja* species

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Authors' contributions

This work was carried out in collaboration between all authors. Author KN designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors HRG and MY managed the analyses of the study. Author MY managed the literature searches. All authors read and approved the final manuscript."

Original Research Article

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ABSTRACT

Aims: This work was for the first time; undertaken to study the effect of different explant types and plant growth regulators on the callus induction and plant regeneration in both *Satureja* species. This protocol can be successfully employed for the large-scale multiplication and conservation of threatened this medicinal plant.
Study Design: The development and maintenance of callus lines from the hypocotyl and leaves of *Satureja hortensis* and *Satureja avromanica* and the study of plant growth regulators on plant regeneration. Micropropagation of these aromatic plants can play a role in the protection of the natural ecosystem, guarantee a massive sustainable production and can provide standardized plant materials for diverse economical purposes.
Place and Duration of Study: Experiments were carried out at the Department of Biology at Razi University of Kermanshah (Iran) in January 2011.
Methodology: The sterilized seeds were cultured on Murashige and Skoog medium, and then the explants were cultured from seedling and transferred to a MS medium supplemented with different concentration of BAP, Kinetin, NAA and 2,4-D growth regulator hormones.
Results: The highest percentage of callus formation frequency (96.67) in *S. hortensis* was

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obtained from hypocotyl explants grown on MS media supplemented with 1 mg L^{-1}2,4-D and 2 mg L^{-1}KIN whereas media supplemented with 0.5 mg L^{-1}2,4-D, 0.5 mg L^{-1}BAP and 0.5 mg L^{-1}NAA was the best for callus formation of hypocotyls (66.67%) in *S. avromanica*. Calli derived from hypocotyl segments of *S. hortensis* showed significantly higher frequency of plantlet regeneration than the calli derived from leaf segments. However, hypocotyl segments of *S. avromanica* were more efficient in plantlet regeneration which produced 87.30% shoot regeneration at MS medium supplemented with 1 mg L^{-1} BA and 1 mg L^{-1} IBA.

Conclusion: This protocol can be successfully employed for the large-scale multiplication and conservation of germplasm these plants.

Keywords: Callus induction; MS medium; Satureja hortensis; Saturejaa vromanica.

ABBREVIATIONS

MS: Murashige and Skoog; BA: N6-benzyladenine; NAA: α-Naphthaleneacetic acid IBA: Indole-3-butyric acid; 2,4-D: 2,4-Dichlorophenoxyacetic acid; Kin: Kinetin.

1. INTRODUCTION

Satureja is a genus of aromatic plants of the family *Lamiaceae*, related to rosemary and thyme [1]. This genus contains 14 species of herbaceous perennial plants that is a year old and has valuable medicinal properties. Savory (*Satureja hortensis* L.) is one of the most important species of the genus *Satureja* that it's antimicrobial and antioxidant properties, has effects on muscle pain, nausea, diarrhea and infectious diseases has proved. Also volatile oil extracted from this plant is especially used in the food industry [2,3].

*Satureja avromanica*is a new perennial, evergreen and non-aromatic species which collected and introduced from West of Iran (Belbar village, Marivan – Paveh strand) recently [4]. It can be used as a newly source of medicinal bioactive compounds of human health benefits. Low rate germination combined with endemic properties of extensive harvesting of *S. avromanica* has resulted in the extinction of this species from regions such as the western portion of the Iran [4]. It is therefore important to develop a protocol for *in vitro* propagation to conserve this medicinally important species from further depletion of its populations, to meet up the demand of the traditional medicine industry [5].

Tissue culture is the process whereby small pieces of explants are isolated from an organism and grown aseptically for indefinite periods on medium under controlled conditions. *In vitro* cultivation of plants is necessary step in a large amount of experiments such as micropropagation, creation of virus free plants, genetic transformation, etc [6]. *In vitro* propagation methods offer highly efficient tools for germplasm conservation and mass multiplication of many plant species [7,8]. Moreover, Plant tissue culture techniques are recognized as useful instruments in crop improvement [9].

There are no reports on an efficient culture system for regeneration in *S. hortensis* and *S. avromanica*. The aim of the present work was to investigate, for the first time, the effect of different explant types and plant growth regulators on the callus induction and plant regeneration in both *Satureja* species.

2. MATERIALS AND METHODS

The experiments were carried out at the department of biology at Razi University of Kermanshah (Iran) in January 2011.

2.1 Plant Material

Satureja hortensis seed was purchased commercially form Pakan Bazr company (Iran) in Junuary 2011. Also, Seeds of *Satureja avromanica* were collected from a Kurdistan: Maryvan to Paveh, Belbar village, N 35º, 14', 22.8" E 46º, 17', 31.2", 830 m. A voucher specimen was authenticated by H. Maroofi and deposited at the botanical garden of the research center of agriculture in Kurdistan, Iran.

2.2 Seed sterilization

Seeds of *S. hortensis and S. avromanica*were surface sterilized with sodium hypochlorite 2% (NaOCl) for 5 min, followed by rinsing three times for 5 min in sterilized distilled water. The seeds were then germinated and grown on MS medium [10] with 2% sucrose and 7% agar. The pH of the medium was adjusted to 5.8 with 0.1 N HCl before autoclaving for 20 min at 121ºC. The cultures were kept in controlled environment at 25±1ºC and 16-8 hr light/dark regime under fluorescent light (19.75 micromole $m^{-2}s$) and 50±10 relative humidity.

2.3 Callus induction

After 2 weeks of culturing, new seedlings sprouting explants were prepared for explants production. According to other studies in the same family [5], explants of hypocotyls and leaves were tested. The explants were aseptically cut into small pieces. Therefore, medium was poured in sterile petri dish and a combination of growth regulators including various concentrations of KIN (0.1, 1 and 2 mg L^{-1}) and 2,4-D (1mg L^{-1}) for *S. hortensis,*, NAA (0.5 and 1.5 mg L^{-1}), BA and 2,4-D (0.5 mg L^{-1}) for *S. avromanica*, and various concentration of IBA and BA were used. The explants were incubated at 25±1ºC under dark and light (1200 lux) condition. After 8 weeks of culture, the percentage of explants producing callus was recorded. The observation was made on the morphology of callus formed in different phytohormones tested.

2.4 *In vitro* Shoot Induction

Well-proliferated calli derived from the leaf segments, 5 weeks after culture, were used for regeneration studies. Approximately 2 grams of fresh callus were placed on the basal MS medium containing 1 mg L^{-1}BA+0 mg L^{-1} IBA, 1 mg L^{-1}BA+0.1 mg L^{-1} IBA and 1 mg L^{-1} BA+1 mg L^{-1} IBA. Data on the mean percentage of shoot formation and shoot length were observed after 12 weeks of culture. Each experiment consisted of 3 replicates. The cultures were incubated at 25±1ºC under an illumination of 1200 lux during a 16/8 h photoperiod obtained from Gro-Lux fluorescent lamps. Well developed plantlets were removed from the culture vessels, washed gently under running tap water and planted in plastic pots containing a potting mixture of sand, soil and farmyard manure in the ratio of 1:1:1. The potted plantlets were covered by polythene bag to maintain suitable humidity. After 30-35 days of bagging, the plantlets were transplanted in the natural condition, where 70% plants were survived.

2.5 Experimental Design and Statistical Analysis

The experiments were done in factorial based on completely randomized design (CRD). Data were analyzed by using SPSS 16. The mean values of different treatments were compared using Duncan's multiple range tests (at 5% level).

3. RESULTS AND DISCUSSION

3.1 Callus Induction in *S. hortensis*

In order to establish the most suitable condition for indirect regeneration, we tried some concentrations and combination of hormones such as KIN (0-2 mg L^{-1}) and fixed concentration of 1 mg L^{-1}2,4-D callus. Callus was optimally induced from leaf segments derived from 2 month old seedling within 28-29 days in darkness and 26-27 days in light of incubation in all treatments containing KIN. Callusing was initiated at the middle of explants and eventually extended all over explants.

The highest percentage of callus induction in *S. hortensis* was 96.67% for hypocotyls explants in the medium consisting of 2 mg L^{-1}KIN, followed by 70% on the medium for having 1 mg L^{-1}KIN+1 mg L^{-1}2,4-D (Table 1). On the other hand, the lowest percentage of hypocotyls callus induction was 40.33% on the medium supplemented with 1 mg L^{-1} 2,4-D+0.1 mg L^{-1} KIN. Analysis of variance (ANOVA) revealed significant differences between growth regulator concentration × explants medium interaction (Fig.1).

Medium supplemented with 1 mg L^{-1}2,4-D+0.1 mg L^{-1} KIN was the optimum concentrations for initiated callus from leaf explants with resulted at 76.66%. With further increase in concentration of KIN there was a low percentage of callus initiation. The lower response (62.33%) in callus formation was observed in basal media supplemented with 1 mg L^{-1}2,4-D+1 mg L^{-1} KIN (Table 1). Callus formed primarily at the margins of green leaf disc after 10 days of culturing but with more callus formation near vascular tissue due to the availability of more nutrients around the conducting tissue.

3.2 Callus induction of *S. avromanica*

The best result in term of percentage response (66.67%) and nature of the callus, in S. *avromanica* hypocotyls explants, were obtained with the combination of 0.5 mg L^{-1} 2,4-D+0.5 mg L^{-1}BA+0.5 mg L^{-1} NAA after 25-30 days (Table 1). NAA concentration decreased significantly callus formation which it reached 40% in medium supplemented with 0.5 mg L^{-1} 2,4-D+0.5 mg L^{-1} BA+1.5 mg L^{-1}NAA.

NAA in combination with BA and 2,4-D induced leaves as well as callus at varying frequencies depending on the concentration and type of explant. In the presence of NAA with BA in the callus induction medium, higher percentage of the explants produced callus (Table 1). The increase of callus formation efficiency was observed with the increase of concentration of NAA. Thus, the best response (33.33%) was observed from leaf segments on MS medium containing 1.5 mg L^{-1} NAA and 0.5 mg L^{-1} BA. A special response of these explants was that low percentage of calluses induction which obtained on the media supplemented with 0.5 mg L^{-1} NAA.

Finally in both studied plants, Explants responded positively when auxins were applied in combination with cytokinins (Table 1). Callus formation was also affected by the source of the explants. Hypocotyl was most effective for callus induction.

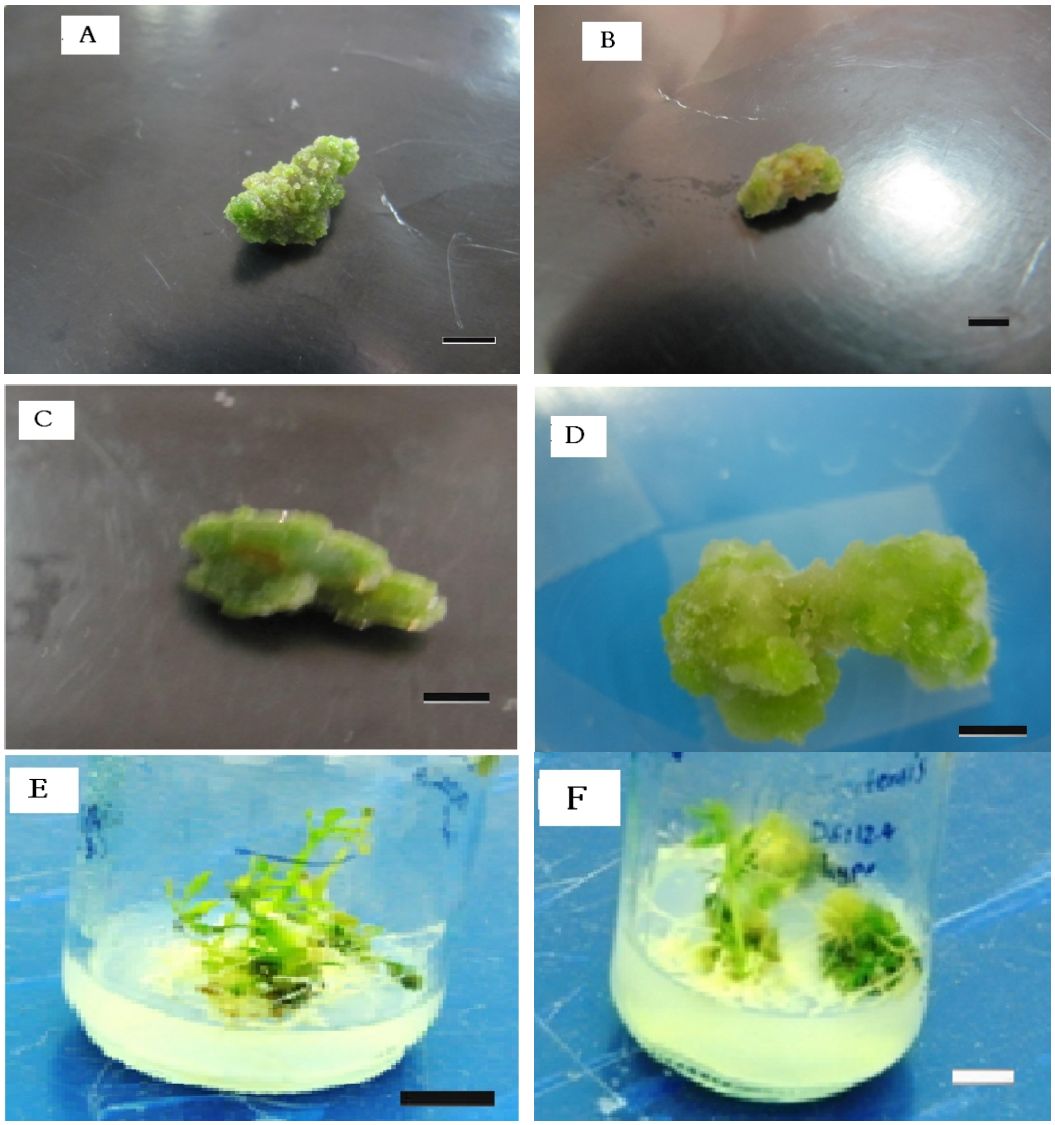

Fig. 1. Callus induction and plant regeneration of *Satureja hortensis* and *satureja avromanica*. a Callus induction in hypocotyls explants of *Satureja hortensis* (bar=1 cm), b Callus obtained from leaves explants of *Satureja hortensis* (bar=0.5 cm), c Callus induction in hypocotyls explants of *Satureja avromanica* (bar=1 cm), d Callus obtained from leaves explants of *Satureja avromanica* (bar=1.5 cm), e Shoot formation from the hypocotyls-derived calli of *Satureja hortensis* (bar=1 cm), f Shoot formation from the hypocotyls-derived calli of *Satureja avromanica* (bar=1 cm)

Table 1. Effects of different concentrations of 2,4-D and NAA alone and in combination with BA or KIN for callus induction from hypocotyl and leaf explants of *Satureja hortensis* and *Satureja avromanica*

Plant	Plant regulators (mg L-1)	Callus induction (%)		Auxins Cytokinins		Explants
	2,4-D	NAA	BA	KIN	Hypocotyl	Leaf
S. hortensis						
	1			0.1	40.33^{ab}	76.66^{c}
	1			1	70^{b}	66.67^{b}
	1			2	96.67^{a}	62.33^{b}
S. avromanica						
	0.5	0.5	0.5	0	66.67^{a}	20^{c}
	0.5	1.5	0.5	0	40^{b}	33.33^{bc}

Value with different letters are significantly different at P = 0.05.

In our experiment, the presence of both 2,4-D and KIN in the medium is necessary for optimum callus formation from hypocotyl and leaf segments of *S. hortensis and S. avromanica* (Table 1). It was found that MS medium supplemented with fixed concentration of 2,4-D (1 mg L^{-1}) in combination with cytokinin (KIN) facilitated callus formation. Similar results were also reported in *Eryngium foetidum* [11] and *Dorema ammoniacum* [12]. Tepe et al. [13] pointed out the significant fluctuations of (BA 1.0 mg L^{-1} and 2,4-D 0.1mg L^{-1} or TDZ 1.0 mg L^{-1} and 2,4-D 0.1 mg L^{-1}) on the amount of callus generated by *Satureja hortensis* L. [13]. There has been limited success in inducing callus in *S. avromanica*, may be due to the initial difficulty in the induction process itself, slow growth rate, low regeneration capacity, and eventual browning of the callus. Browning of the callus in some cases, may be due to the activation of secondary metabolite synthesis [14].

3.3 Shoot Regeneration in *S. hortensis*

The calli derived from various explants were subcultured on MS medium supplemented with BA (1 mg L^{-1}) alone or in combination with IBA (0.1 to 1 mg L^{-1}) for shoot induction (Table 2). Two week after transfer to regeneration medium, sub-cultured calli enlarged rapidly and green spots appeared on the surface. Green shoot buds started emerging on the various calli on the 5th weeks. The regeneration frequency and the number of shoots per callus varied in hypocotyls and leaf cuttings derived calli. Shoot regeneration from the calli was determined by the type of growth regulator, its concentration and combination. Interestingly, calli derived from hypocotyl segments of *S. hortensis* showed significantly higher frequency of plantlet regeneration than the calli derived from leaf segments. Therefore, MS medium supplemented with 1 mg L^{-1}BA and 1 mg L^{-1} IBA produced the highest frequency of shoot regeneration (72.3%) in hypocotyl-derived callus (Table 2).

3.4 Shoot Regeneration *in S. avromanica*

Shoot initiation appeared from hypocotyl and leaf derived calli within ten days of subculture. The highest percentage of shoot induction in leaf segments was 63.23% on MS medium supplemented with 1 mg L^{-1}BA and 1 mg L^{-1}IBA followed by 27% on the medium consisting of 1 mg L^{-1}BA and 0.1 mg L^{-1}IBA. The hypocotyl regeneration callus was 87.30% on the medium having 1 mg L^{-1}BA+1 mg L^{-1} IBA followed by 26.67% on the medium consisting of 1 mg L^{-1}BA+0.1 mg L^{-1}IBA. Therefore, MS medium supplemented with 1 mg L^{-1} BA and 1 mg

L^{-1} IBA produced the highest frequency of shoot regeneration (87.3%) in hypocotyl- derived callus (Table 2).

To the best of our knowledge this is the first report on the callus induction and regeneration from hypocotyl and leaf explants in *S. hortensis* and *S. avromanica*. Traditional approaches to plant regeneration from calli by manipulating the relative ratio of auxin to cytokinin have been successfully used in the current investigation [15].

In the present study, BA alone or a combination of auxins and BA was essential for the regeneration of the calli. Superiority of BA for shoot induction has been reported to be due to the ability of plant tissue to metabolize natural hormones more readily than artificial growth regulators or due to the ability of BA to induce production of natural hormones such as zeatin with in the tissue and thus work through natural hormone system [16]. Existing reports suggest that auxins at lower concentrations along with cytokinins have a critical role in plant regeneration in several systems like *Petasites hybridus* [17], *Eucalyptus grandis* [18], *Hybanthus enneaspermus* [19], *Coleus forskohlii* [20], *Saccharum spp*. [21] and *Eleusine indica* [12].

The regeneration frequency of shoots per callus clump varied in callus produced from various explants. Hypocotyl-derived calli showed better response than the calli produced from leaf cuttings in both studied species. Variation between explants within a clone was a common phenomenon in some medicinal plants like *Eryngium foetidum* [11], *Dorema ammoniacum* [12] and *Cardiospermum halicacabum* [15]. Similar to our observations, Tawfik and Noga [14] noticed that 2652ptimizati responded better than other cumin seedling explants for proliferation.

This is the first report on callus induction and regeneration protocols of *S. hortensis and S. avromanica*, which will provide a valuable tool in an improvement program. Callus with regeneration potential reported in this study could be useful in raising regenerable suspension cultures and isolation of totipotent protoplasts. The described method can be successfully employed for conversion of germplasm and the large-scale multiplication of both *Satureja* species as valuable medicinal plants. Further work is needed to maintain the capacity of plant regeneration from callus cultures in order to employ this callus as experimental material.

Table 2. Effect of different concentrations of BA alone or in combination with IBA on regeneration of adventitious *Satureja hortensis* and *Satureja avromanica* shoot from callus produced from hypocotyls and leaves explants (Medium: MS; culture period: 6 weeks)

Plant	Growth regulators (mg L^{-1})		Hypocotyl shoots (%)	Leaf shoots (%)
	BA	IBA		
Satureja hortensis				
	1	0	0^{b}	0^{b}
	1	0.1	0^{b}	0^{b}
	1	1	72.30^{a}	13.20^{b}
Satureja avromanica				
	1	0	0^{b}	0^{b}
	1	0.1	26.67^{b}	27^{b}
	1	1	87.30	63.23^{b}

Value with different letters are significantly different at P = 0.05.

4. CONCLUSIONS

Significant progress has been made in the *In vitro* regeneration systems of many traditional medicinal plants. The experimental results recorded from our experimental research aimed at investigating the possibility of callus induction and plant regeneration of *S. hortensis* and *S. avromanica*, revealed the following:

- *In vitro* modeling of explants (leaf and 2653ptimizati) in order to obtain callus under the influence of exogenous phytohormones (KIN, NAA, IBA and 2,4-D), is possible;
- Explants that generated the largest quantities of plant callus were mainly those of 2653ptimizati or 2653ptimiz type;
- KIN exogenous phytohormones generated the largest quantities of plant callus, regardless of the explant type; the amount of callus obtained from *In vitro* modeling of explants is dependent on both the content of exogenous phytohormones and on the explant type. These are novel methods of conserving the natural populations of medicinal plants, reducing the risk of their extinction.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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