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VEGETATIVE MULTIPLICATION OF *SALVIA OFFICINALIS* L. FOR THE OBTAINING OF TRUE-TO-TYPE PLANTS

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INTRODUCTION

Salvia officinalis L. is an important specie of plants with multiple uses. Sage generally grows about a foot or more high, with wiry stems. The leaves are set in pairs on the stem and are 1 1/2 to 2 inches long, stalked, oblong, rounded at the ends, finely wrinkled by a strongly-marked network of veins on both sides, greyish-green in colour, softly hairy and beneath glandular. The flowers are in whorls, purplish and the corollas lipped. They blossom in August. All parts of the plant have a strong, scented odour and a warm, bitter, somewhat astringent taste, due to the volatile oil contained in the tissues.

Sage has a very long history of effective medicinal use and is an important domestic herbal remedy for disorders of the digestive system. Its antiseptic qualities make it an effective gargle for the mouth where it can heal sore throats, ulcers etc. The leaves applied to an aching tooth will often relieve the pain. The whole herb is antihydrotic, antiseptic, antispasmodic, astringent, carminative, cholagogue, galactofuge, stimulant, tonic and vasodilator. Externally, it is used to treat insect bites, skin, throat, mouth and gum infections and vaginal discharge.

Vegetative multiplication of *Salvia officinalis* L. can be achieved by„in vitro" tissue cultures with high impact on the breeding activity of this specie. The advantages of this unconventional method lays onits inreased multiplication outturn, aspect associated with also with the assurance of a high degree of uniformity and conformity of the obtained descendences. Comparing with the classical methods, the tissue culture "in vitro" presents the following advantages: the process is not limited by the seasonal changes imposed by the succession of seasons, the control of growth regulators is assured, it prevents the manifestation of negative influences determined by the expression of genetic variability in descendences, the genetic conservation of selected plants is assured for a longer period of time.

An extremely important stage for the establishment of an efficient multiplication method "in vitro" is the determination of optimum conditions for the induction, support and development of regenerative processes of cultivated explants. The factors that influence the capacity of regeneration of plants "in vitro" are numerous, but among them, the most important are the exogenous growth regulators.

This paper describes the first results of experiments carried out to induce organogenesis in tissue culture of *Salvia officinalis* L. under influence of different combinations of growth regulators. It also aims toward the identification of explant type, as well as the cultivation media and environmental factors that are optimal for the regeneration of plants with the same genetic inheritance.

MATERIAL AND METHODS

The biological material used in our experiments Belongs to Vegetable Research and Development Station Bacau.

For the purpose of multiplication of selected genotypes the donor explants were harvested to ensure maximum reactivity in terms of multiplication yield, as well as maintaining the safety of the original genotype. The explants used in our experience consist apex explants type (vegetative peak) and uninodal explant.

Harvesting of explants was performed on the mother plant, healthy, maintained under controlled environment greenhouse. Ensuring optimal conditions of light, temperature, humidity, photoperiod for mother plants proved to be a primary condition for the further development of explants cultured "in vitro" in buds and shoots, having a big influence on their performance of multiplication.

The explants were surface sterilized with 0.1% (wt/vol) mercuric chloride ($HgCl_2$) for 7 minutes, followed by repeated washing with sterile distilled water. Seeds were germinated in the dark in full strength of basal medium Murashige and Skoog, 1962, supplemented with 3% sucrose and 0.8% agar.

Under aseptic conditions, explants were inoculated on basal MS (Murashige and Skoog, 1962) medium containing 3% (w/v) sucrose, supplemented with different concentrations and combinations of 6-benzylaminopurine (BA) indole-3-acetic acid (IAA) or naphtyl acetic acid (NAA) and zeatin for direct plant regeneration – table 1.

Table 1. Components of different nutrient media for shoot induction at *Salvia officinalis* L.

	V1	V2	V3	V4	V5	V6
BAP	3,0 mg/l	2,0 mg/l	1,0 mg/l	-	5,0 mg/l	3,0 mg/l
Kin	-	-	-	1 mg/l	-	-
NAA	0,5 mg/l	-	-	0,1 mg/l	1,0 mg/l	1,0 mg/l
IAA	-	-	-	-	0,1 mg/l	-
Sugar	3%	3%	3%	6%	3%	6%
pH	5,8	5,8	5,6	5,6	5,8	5,6
Agar	8 ‰	8 ‰	8 ‰	8 ‰	8 ‰	8 ‰

The pH was adjusted to 5.8 prior to the addition of 0.8% agar and autoclaved at 121°C (1.06 kg/cm^2) for 25 min.

The cultures were incubated in culture chambers with controlled light, humidity and temperature control at 25^{0}C, a 16-h photoperiod, and 5000 lx light intensity. Repeated sub cultures were done at an interval of 30 days and incubated under the same temperature as mentioned previously. The culture vessels showing signs of contamination were discarded. Day to day observation was carried out to note the responses.

The elongated shoots were cut out and transferred into rooting medium that contained auxins in different concentrations (NAA and IAA) – table 2.

Table 2. Components of different nutrient media for root induction at *Salvia officinalis* L.

	M1	M2	M3	M4	M5
NAA	0,8 mg/l	0,7 mg/l	0,6 mg/l	-	-
IAA	-	-	-	0,6 mg/l	-
IBA	-	-	-	-	0,6 mg/l
ZAHAR OZĂ	3%	3%	3%	3%	3%
Agar	8 ‰	8 ‰	8 ‰	8 ‰	8 ‰
pH	5,8	5,8	5,8	5,8	5,8

The rooted plantlets were washed and transferred to the hydroponics conditions in bottles. Different variants were tested: simple water, addition of Previcur (a substance used to control fungus) in concentration of 0.15% and directly to potting mixture in plastic pots. The pots were covered with clear bags to provide 100% relative humidity. They were placed in an acclimatization room under a 16/8 h photoperiod at 20 - 23°C.

The acclimatized plants were planted in a potting mixture of sterilized sand + vermiculite (1:1 ratio) in plastic cups, hardened in a mist chamber (80% relative humidity) for 2 weeks before transfer to green house. The plants were allowed to grow to maturity. They normally passed vegetative phase.
For each genotype, 10 hypocotyls were cultured and the experiment was repeated 3 times.

The percentage of buds forming regeneration structures and the mean number of shoots per explant was recorded. The data were analyzed by ANOVA (analysis of variance). The means were compared using the Duncan multiple comparison test at $P < 0.05$. In tables the mean values and standard error for each genotype were shown.

RESULTS AND DISSCUSSIONS

For the multiplication of *Salvia* plants were used as initial explant apexes and uninodale explants harvested from mother plants who had exceptional qualities which were grown under controlled greenhouse.

Immediately after inoculation explants significantly increased their volume and peripheral areas showed slight traces of necrosis. Observations on the initiation, development and multiplication of shoot were done regularly at intervals up to one week.

The initiation of regenerative processes could be observed after about 10-14 days on the explants. In case of apex explants type, on observed an increase in their length as well as on foliar weight and the appearance at the base of the explant of meristematic areas generating shoots.

Uninodale explants also showed a slight increase in volume, followed by the emergence of 1-2 shoots generated from the internode.

Not all the explants inoculated had the same reaction morphogenetic explants knowing that there are functional differences even between morphologically similar explants. At this stage of ontogenetic development there are particularities of the reaction in terms of their ability to form organs, features that rely precisely on its own totipotentiality of each explant.

The reaction of the explants on the 6 types of induction medium used in our experiments is shown in Table 3.

Table 3. The frequency of morphogenetic response "in vitro" of explants on media with different PGRs

Type	Reactive explants	V1	V2	V3	V4	V5	V6
Apexes	217	62	7	13	-	79	56
Uni-nodale explants	188	27	-	11	-	63	45

*Mean and standard deviation. Each treatment had 30 explants.

Of the 300 explants inoculated 217, 188showed that morphogenetic response, the rest is just

continued to grow slowly in length (not proliferate new shoots) or degenerated by necrosis, or were removed due to infection.

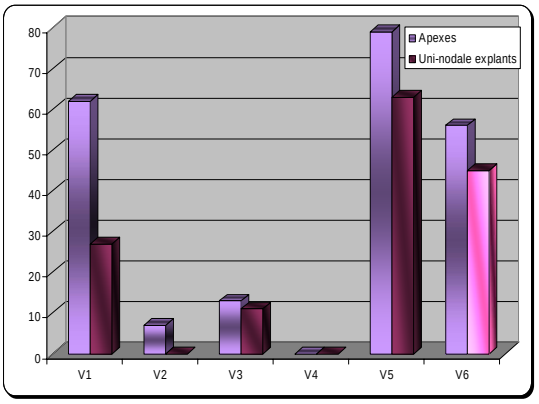

Fig. 1. Graphical representation of the main morphogenetic reactions of *Salvia officinalis*

Among the 6 variants of culture medium tested three of them (V1, V5 and V6) have led to a better response morphogenetic, two (V2 and V3) allowed induction of regenerative processes but in a small percentage and only one not allowed the obtaining of meristematic centers. It is well known that directing morphogenetic processes towards the development of shoots directly on explant can be achieved by adding to the basic culture media different types of cytokinins. In our experimentation impact tests were carried out on two of the most important cytokinins, BAP and kinetin. The results obtained (Table 3, Figure 1) showed that from the two types of cytokinins used, the greatest efficiency in generating shoots has BAP (as demonstrated by other authors Gymnema sylvestre - Komalavalli et al., 2000 Hyptis suaveolens - John Britto, 2001). Each of the three different initiation medium that allowed meristematic structures are characterized by the presence of BAP in combination with the hormone NAA, as well as IAA (V5 variant). Replacing BAP with kinetin (V4 variant) does not allow the apparition of embryogenic structures. This highlights the beneficial influence of BAP compared with other cytokinins.

After almost 7-8 days the shoots were transferred on fresh media that should support the regenerative processes. Regeneration of shoots was achieved either by adventitious shoots formation (at base explants inoculated) and by the development of pre-existing meristematic centers. Part of regenerated shoots was transferred on fresh culture media for further regenerative processes. Depending on the way that plantlets evolved, they were transferred either on a rooting media or directly to hydroponic conditions. The plants that presented a well developed rooting and foliar system were transferred directly in hydroponic conditions for their acclimatization.

Based on our previous experience regarding the beneficial effect of the auxin NAA on the effectiveness of rooting processes we proceeded to test its gradual concentration in three variants of rooting medium, while IAA and IBA were used only in the amount 0.6 mg/l. The number of shoots that formed roots on the culture media are shown in Table 4 and figure 2.

Table 4. The morphogenetic reaction of shoots on the variants of rooting medium

Nr. crt.	Variant of medium	% rooting
1.	M1	88.5
2.	M2	83.8
3.	M3	96.2
4.	M4	63
5.	M5	51.4

Among the five variants tested, the best response was observed in case of variant M3, which is characterized by the presence of auxin NAA in an amount of 0.6 mg/l. M1 and M2 variants, although it led to the formation of fairly large proportion of the roots - 88.5, respectively 83.8 have failed to reach the threshold of 96.2% (rate achieved by the variant M3).

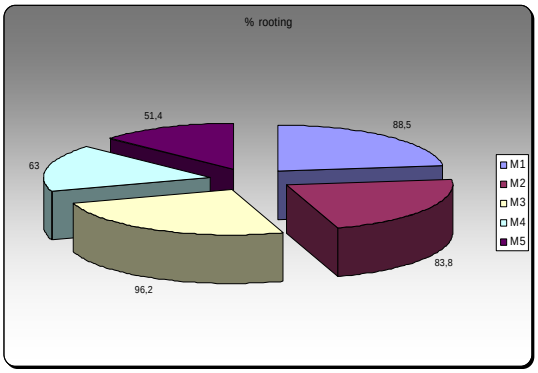

Fig. 2 – Graphical representation of the morphogenetic reaction of shoots on the variants of rooting medium

Regenerated plants with six or eight leaves were transferred on hydroponics medium and kept about four days covered with a plastic foil, in the culture room. Subsequently, they were day by day acclimatized to room atmosphere. Surviving plants were transferred to the greenhouse and grown to maturation.

CONCLUSIONS

The results of this study showed that *Salvia officinalis* L. is a species suitable for cultivation under "in vitro" conditions. The morphogenetic explants reaction is positive, the initiation and development of regenerative structures leading to neopropagule formation.

The incubation of the cultures in photoperiod of 16 hours light/ 8 hours dark, with a light intensity of 3000 lx and a temperature of 25ºC +/- 1ºC represents the best condition for the initiation, development and regeneration of plants.

Under the experimental conditions tested apexes used as initial explant allowed uniform and stable plant regeneration.

The results obtained showed that from the two types of cytokinins used, the greatest efficiency in generating shoots has BAP. Replacing BAP with kinetin does not allow the apparition of embryogenic structures.

The highest percentage of rooting was obtained on M3 variant, characterized by the presence of NAA hormone in the amount of 0.6 mg/L. Increasing the amount of auxin has led to an increase in the percentage of rooting, but on the contrary, its values decreased as the concentration increased.

The experimental data obtained so far encourage further research to determine all the factors that impact on regeneration processes (genotype, explant, etc.) having as purpose to establish a quick and efficient method of multiplication "in vitro", which ensure a short time to an optimum number of plants with the same hereditary as mother plants.

ABSTRACT

Multiplication „in vitro" is used routinely to generate a large number of high-quality clonal agricultural plants, including ornamental, medicinal and vegetable species. Micropropagation has significant advantages over traditional clonal propagation techniques. These include the potential of combining rapid large-scale propagation of new genotypes, the use of small amounts of original germplasm (particularly at the early breeding and/or transformation stage, when only a few plants are available), and the generation of pathogen-free propagules.

This paper describes the first results of experiments carried out to induce organogenesis in tissue culture of *Salvia officinalis* L. under influence of different combinations of growth regulators. It also aims toward the identification of explant type, as well as the cultivation media and environmental factors that are optimal for the regeneration of plants with the same genetic inheritance.

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