ADVENTITIOUS MERISTEM ORGANOGENESIS AND SHOOT PROLIFERATION OF *SALVIA OFFICINALS* L. IN SPLIT-NODAL CULTURE

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Abstract : Nodal segments were prepared from the stems of in vitro propagated salvia plants. The explants were then cut longitudinally, through the sites of the dormant axillary buds into two halves. The responses of the split-nodal explants to dark initial incubation, thidiazuron (TDZ) and to benzyladenine (BA) alone or plus TDZ in MS (Murashige and Skoog, 1962) medium were studied in sequential consecutive experiments. In the first part of the study, the explants were cultured on the medium either lacking (control) or containing 1 µM to 4 µM TDZ. The cultures were kept either directly under light or after receiving a week of incubation in the dark. The data showed that more shoots were regenerated from the cultures that received the dark pretreatment than those maintained directly under light. Existence of 2 µM TDZ was the optimum concentration to regenerate normal shoots of which high percent (85%) formed roots on secondary basal medium [with 5 µM indole-3-butyric acid (IBA)] and ex vitro survived (74%). Subsequently, the addition of BA (0 to 3 µM) to the medium containing 2 µM TDZ was investigated. The greatest number of shoots per explant was obtained from the cultures on TDZ (2 µM) containing medium when supplemented with 2 or 3 µM BA. This study presents a new results suggesting a beneficial use of dark pretreatment, TDZ and BA plus TDZ in tissue culture of salvia. The utilization of split-nodal explants may be useful in both micropropagation and in enabling the introgression of desirable foreign gene(s) into salvia genome, especially via *Agrobacterium*.

Introduction

Salvia spp. could be propagated in vitro via shoot proliferation from axillary buds of intact stem nodal sections on medium with benzyladenine (BA) (Hosoki and Tahara, 1993; Mederos-Molina et al., 1997, Tawfik and Noga, 2001) but the shoot multiplication rate is usually low. During the last decade, on the other hand, a strong cytokinin-like effects of thidiazuron (TDZ) has been consistently reported in tissue culture of several other plant species (Babaoglu and Yorgancilar, 2000, Fiola et al., 1990) including

ornamental (Henny and Fooshee, 1990; Andrade et al., 1999) and medicinal plants (Li et al., 2000) TDZ is used to defoliate mature green leaves of cotton before harvest (Henny and Fooshee, 1990). At low concentrations, it shows a cytokinin activity (Fellman et al., 1987). TDZ has been referred to as "cytokinin-like compound" because it does not have the purine structure that characterize the conventional cytokinins

In contrast to BA, thidiazuron has been shown to be several folds more effective in enhancing in vitro adventitious shoot initiation and proliferation (Cuenca et al., 2000; Fiola et al , 1990. Sriskandarajah et al., 2001) Furthermore, recent studies suggested that combination of TDZ and BA is more effective than TDZ alone (Khalafalla and Hattori, 1999; Kim et al., 1997) In addition to the supplements of plant growth regulators (PGR) in the medium, dark pre-incubation of explants is reported to enhance the process of adventitious meristem initiation and shoot proliferation (Leblay et al., 1991. Nehra and Stushnoff, 1989. Sriskandarajah et al., 2001) There is a need to study the responses to these PGR supplements and explant pre-treatments of in vitro cultures of salvia as being an important spice and medicinal plant for which there has been no reliable shoot regeneration protocol.

While intact nodes are suitable for micropropagation, wounding the explant area of competent tissues stimulates adventitious meristem organogenesis (McClean and Grafton, 1989) Wounded tissues also is required in the production of genetically engineered plants especially when the introduction of the foreign gene(s) is mediated via *Agrobacterium* If shoot regeneration occurs from a wounded excised explant without an intermediate callus phase, the chance to induce variants is reduced (Evans and Bravo, 1986). Thus preparation of nodal tissues for plant regeneration in such a way would be useful in both micropropagation and the application of the molecular based manipulations of gene transfer for improvement of salvia. The present study, therefore, was implemented to investigate the regeneration of salvia plants from split-nodal explants in response to dark pretreatment, different concentrations of TDZ and combinations of BA and TDZ.

Materials and Methods

I- General procedures

I-A. Explant source axenic-plants

Salvia shoots, 5-7 cm long, were detached from 6-month-old plants. These shoots were sectioned into cuttings of single nodes The nodal explants were then surface sterilized with calcium hypochlorite [Ca ($Cl O)_2$]. About 0.5 g/l Triton X-100,

wetting agent, was added to the calcium hypochlorite. The explants were stirred for 15 min in this sterilizing solution. Then the explants were rinsed 4 times in sterilized distilled water under aseptic conditions and were blotted to dry on a sterilized filter paper before incubating them in 200 ml baby food jars containing 25 ml nutrient medium. The medium was prepared according to the MS (Murashige and Skoog, 1962) recipe. It contained 30 g/l sucrose and 8 g/l agar. The pH of the medium was adjusted to 5.8 before autoclaving at 120° C under 1.2 $kg.cm^{-2}$. The nodal cultures were incubated for 4 weeks at 23° C under cool fluorescent light ($40\mu mol.m^{-2} s^{-1}$ 16h/day). Proliferated axillary shoots were repeatedly excised and sectioned into nodal cuttings. These nodal explants were subcultured on fresh medium to produce and maintain sufficient supply of axenic explant-source material.

1-B. Preparation of nodal segments

The explant used in this study was split-nodes (SN). This explant was the excised stem nodes of the axenic plants after being cut, passing through the axillary bud sites on the both sides of the node, into two longitudinal halves. The prepared explant consisted of the nodes attached to 2-3 mm stem internodal portions. The sites of the dormant axillary buds were gently scraped to remove organized buds and pre-existing meristems. The split-nodal segments were cultured with their cut surface contacting the medium. Investigation of adventitious shoot regeneration from these explants was conducted utilizing MS medium supplemented with thidiazuron (TDZ) and benzyladenine (BA) alone or plus TDZ in sequential consecutive-experiments.

II- Specific study

II-A.. Explant culture on medium with thidiazuron (Expt. I)

In this experiment, the medium (shoot regeneration medium, SRM) was utilized lacking (control treatment) or containing 1, 2, 3 and 4 µM TDZ. Two experiments were carried out. In the first experiment, the explants of the SN were incubated for a week in darkness followed by 4 weeks under light. In the second one, the SN were kept during the whole 5 weeks under illumination without the dark pre-treatment. The temperature and light conditions were the same as indicated above. The experimental design was randomized complete-blocks (RCBs) with four replicates. One SN explant was cultured in each baby food jar. Twelve jars were used for each treatment per replicate. Samples of one SN explants per replicate were taken at the end of the first and the third weeks of the incubation for anatomical analysis.

The percentage of the explants regenerating shoots (responded explants) and the vitrification rate (%) were determined 4 weeks after the culture. Subsequently, the explants were subcultured on MS medium lacking TDZ for shoot elongation. The total number of the regenerated normal-shoots (at least 1 cm long) was determined after 4 weeks of the subculture The harvested normal shoots (non-vetrified) were rooted on medium with 5 µM indole-3-butyric acid (IBA). The rooting was a RCB experiment corresponding to the preceding treatment of the SRM The percentage of the rooted shoots and the number of roots formed per plant were determined Rooted shoots were transplanted in plastic pots containing a mixture of sand and peat moss (1:1, v/v). The transplants were watered with half-strength Hoagland nutrient solution The pots of the transplants were kept in acclimatization boxes for 10 days. The plants were gradually acclimatized to the ex vitro conditions and the survival rate was calculated.

II-B. Explant culture on medium with benzyladenine (BA) alone or plus TDZ (Expt. II)

SRM were prepared containing 1 or 2 or 3 µM BA alone or plus 2 µM TDZ. The SRM with 2 µM TDZ alone was used as reference treatment Thus this experiment had seven treatments. The concentration of 2 µM TDZ was chosen based on preliminary observations on the nodal explant responses to the TDZ in the abovementioned experiment (testing different supplemented levels of TDZ). The experiment was arranged in RCBs with four replicates. The cultures were kept in darkness for a week followed by four weeks under light. Otherwise, the incubation conditions and the experimental procedure including data records, rooting, and acclimatization were the same as indicated elsewhere above in Expt I.

III- Histological procedure

Explant samples for histological analysis were immediately immersed in fixing solution (FAA) The FAA solution composed of a mixture (10: 1: 2, by volume) of ethanol (100%), glacial acetic acid and formalin (40%) plus 7 volume parts of water. Following dehydration in ascending ethanol series, the material was infiltrated and then embedded. Microtome sections were cut 15 µm thick and mounted on glass slides. These sections were stained with safranin and then in fast green.

IV- Statistical procedure

In both experiments, the data were subjected to a combined analysis of variance (ANOVA) (Gomez and Gomez, 1984). For the

vitrification rate, the square root transformed data were used for the ANOVA Otherwise, the original data were used. Those data of the shoot regeneration on medium with different concentrations of TDZ were combined over years (2000 and 2001) and culture conditions (dark pre-treatment and the culture without receiving this treatment). The data of the shoot regeneration on medium with benzyladenine (BA) alone or plus 2 µM TDZ, were combined over years. Years and replicates were considered random effects in both experiments The 'Least significant Differences' (LSD) were calculated at 0.05 level of probability to separate differences between means of the TDZ concentration in the first experiment. Dunnett's test was used to compare all treatment (BA and BA plus TDZ) with the medium containing 2 µM TDZ (reference treatment) in the second experiment (Steel and Torrie 1980) LSD at 0.05 level of probability was also calculated to compare two means of BA and BA plus TDZ when needed.

Results and Discussion

I-General developmental responses

Single shoots developed from the wounded tissues of one side of some explants (10-20%), on the medium lacking TDZ and BA supplements. Such shoots grew slowly On the contrary, the cultures of intact nodes (Tawfik and Noga, 2001) readily produced single shoots on both sides of 90-100% of the explants. Obviously, these contradictory responses occurred as a consequence of the interruption of the integrity of the pre-existing nodal meristems as a result of splitting the explant in the present study.

On the other hand, after a week of incubation on the SRM with TDZ (Expt.I) and also when BA alone or plus TDZ (Expt.II) was added. enlarged tissues were observed on the place of the wounded axillary regions of the SN explants They were pale green and with thickness of about 3-4 mm in the cultures incubated in darkness In those cultures kept under light, the enlarged tissues were dark green and 1-2 mm thick. While the enlarged tissues developed from the wounded axillary sites of the nodes, the attached internodal tissues of the explant turned brown. Histological observations indicated the existence of several distinct meristem initials (Fig. 1A and B) in these enlarged tissues after a week of the culture. Differentiated leaves were found in the third week of the culture (Fig. 1C and D). Multiple shoots and shoot-buds developed on both sides of the SN during the fifth week after culturing (Fig. 2A). These results indicate that TDZ and BA alone or plus TDZ were necessary to initiate new meristems from the

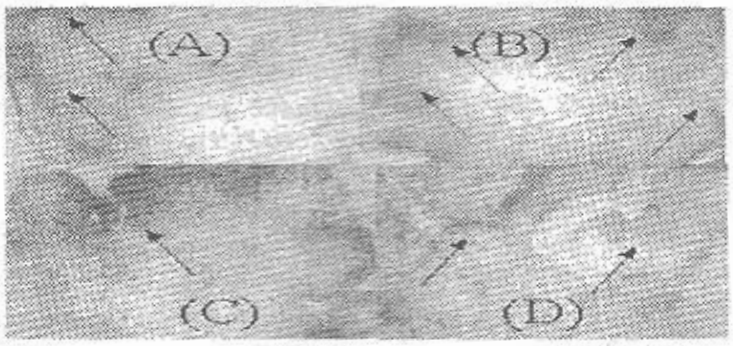

Fig. (1): (A and B) Longitudinal sections in the expanded axillary tissues developed on the wounded nodes of salvia split-nodal explants a week after incubation in light and darkness, respectively, on MS medium containing 2 µM thidiazuron (TDZ) and benzyladenine (BA); notes the initiation of several adventitious meristem sites (arrows). (C and D) Longitudinal sections showing differentiated leaves after 3 weeks of the culture.

competent tissues in the place of the removed pre-existing meristems. Shoot elongation occurred 2 weeks after transferring the multiple-shoots onto the medium lacking plant growth regulator (Fig. 2B). New excisable shoots could be harvested, subsequently, at 5-7 day-intervals. The cut shoots formed roots and grew producing 5 to 7 leaves (Fig 2C) on rooting medium with 5 µM IBA. The plants were acclimatized (Fig. 2D) to the ex vitro conditions (Fig. 2E)

II- Quantitative evaluation of the explant responses

The existence of similar trends in both years was revealed by lack of significance of the variance due to the interaction between the treatments and years. There were no significant differences between the years in both experiments, therefore, the data were pooled over years (Table 1 and 2; Fig. 3).

II-A.. Cultures on medium with thidiazuron (TDZ (Expt. I)

Plant regeneration of salvia was not significantly affected by the interaction between the dark pretreatment and the different concentrations of TDZ. Only the main effects due to these factors, therefore, are presented in Tables 1 and 2.

II-A-1. Light vs. dark pretreatment: More shoots were regenerated on explants received a week of dark pretreatment (Table 1) during the incubation on the SRM supplemented with TDZ. The percentage of responded explants and the rate of vetrification were not significantly affected. Whether or not the explants received the dark pretreatment, the rooting response of the regenerated shoots did not

influence. Also the survival rate of the produced plantlets did not significantly differ (Table 1). These results suggest that the beneficial effect of the dark pretreatment in increasing the number of the regenerated shoot from the SN cultures of salvia resulted, most likely, from increasing the number of the newly induced meristematic

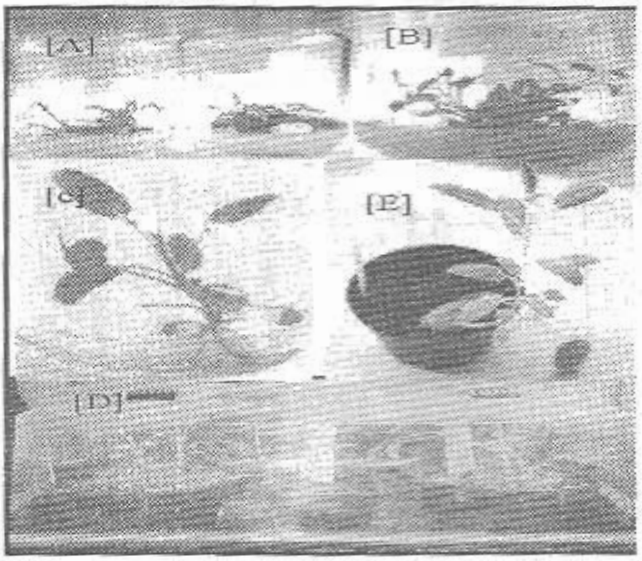

Fig.(2): Plant regeneration of salvia from the culture of split-nodal explants: (A) Multiple shoots grew on both sides of the nodal sites after 5 weeks of incubation on MS medium with 2 μM thidiazuron (TDZ) and benzyladenine (BA), (B) Shoot elongation 2 weeks after subculturing onto the medium lacking TDZ and BA, (C) Plantlets obtained from the cut shoot when transferred into rooting medium containing 5 μM indole-3-butyric acid (IBA), (D) Transplants in acclimatization boxes, and (E) Plants after acclimatization to the ex vitro conditions

regions from the competent explant tissues. Similar enhancement effect of dark pretreatment was noticed by researchers in pear (*Pyrus communis* L.) (Leblay et al., 1991) and watermelon (*Citrullus lanayus* Thumb) (Compton, 1999). About 3 folds increase in the number of regenerated shoots was obtained by dark pretreatment in the *Campanula carpatica* Jacq. cultures of different explant types (Sirskandarajah et al:., 2001). Initial dark pretreatment is suggested to enhance the process of new meristem initiation. This treatment could avoid photoinactivation of the endogenous plant hormones during the critical initiation phase of the meristems

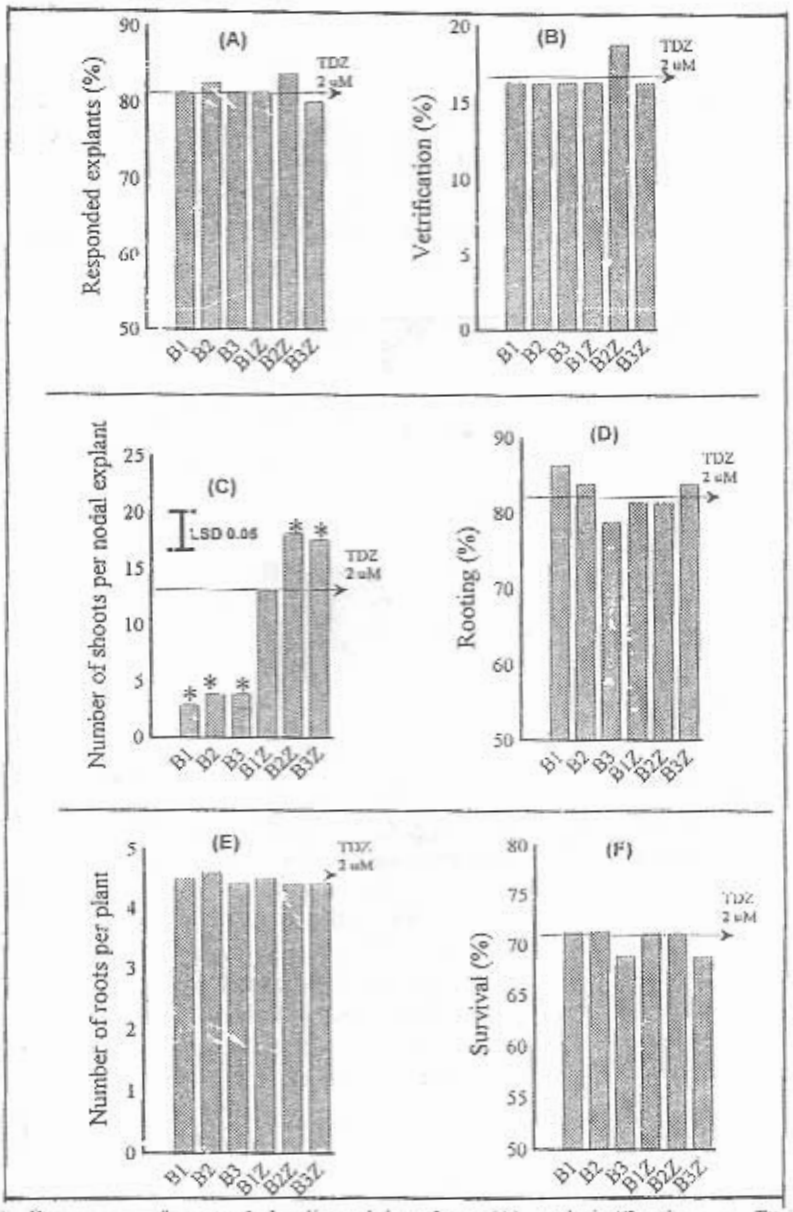

Fig.(3): Percentage of responded split-nodal explants (A), and vitrification rate (B) in the cultures on shoot induction MS medium with different concentrations of benzyladenine (BA) alone or plus 2 µM thidiazuron (TDZ) for 5 weeks as compared with the medium containing 2 µM TDZ alone (reference medium, the top arrow-headed horizontal line). In (C) is the number of shoots harvested per the explant during 4 weeks of incubation on the medium lacking TDZ and BA while the average rooting percentages and the number of roots formed per shoot on rooting medium with 5 µM indole-3-butyric acid (IBA) are shown in (D) and (E). The average survival rate is presented in (F). Data are averages of two years (2000 and 2001). Stars denote significant deviations from the reference medium using Dunnett's test at 0.05 level of the probability. Vertical lines are the "Least Significant Differences" to compare two means of the BA and the BA plus TDZ treatments at 0.05 level of the probability

Table (1): Plant regeneration of salvia from the culture of split-nodes as affected by the dark vs. light pre-incubation treatments.

Pre-incubation treatment [a]		Induction/proliferation Medium [b]			Rooting Medium [c]		Ex Vitro
		Responded explants (%)	Vitrification (%)	Shoots/ node (no.)	Rooting (%)	Root (no.)	Survival (%)
Dark	2000 [d]	81.3	15.0	10.8	81.9	3.8	65.0
	2001	79.4	16.8	12.1	85.0	4.0	66.3
Average		80.4	15.9	11.5	83.3	3.9	65.7
Light	2000	79.4	16.3	6.6	81.9	3.4	64.4
	2001	84.4	20.0	7.1	79.4	3.8	68.1
Average		81.9	18.2	6.8	80.7	3.6	66.3
Dark vs Light		*ns* [e]	*ns*	** [f]	*ns*	*ns*	*ns*

[a] Cultures were maintained either directly under cool white light (16h/d) or after a week of pre-incubation in darkness.

[b] Contained thidiazuron (TDZ).

[c] Contained indole-3-butyric acid (IBA).

[d] Variance due to year and its interaction with pre-treatments were not significant.

[e, f] Non-significant and significant ($P < 0.05$), respectively.

Table (2): Plant regeneration of salvia from the culture of split-nodes as affected by the concentration of the thidiazuron (TDZ) in the induction/proliferation medium.

TDZ (µM)		Induction/proliferation Medium[a]			Rooting Medium [b]		Ex Vitro
		Responded explants (%)	Vitrified shoots (%)	Shoots/ node (no.)	Rooting (%)	Root (no.)	Survival (%)
1µM	2000[c]	82.5	11.3	6.5	86.3	4.7	68.8
	2001	86.3	13.8	7.0	82.5	4.8	70.0
Average		84.4	12.6 (3.5)[d]	6.8	84.5	4.8	69.4
2µM	2000	82.5	13.8	10.1	86.3	4.8	70.0
	2001	81.3	17.5	11.5	85.0	4.5	75.0
Average		81.9	15.6 (3.9)	10.8	85.7	4.7	72.5
3µM	2000	76.3	16.3	9.3	83.8	3.1	61.3
	2001	82.5	18.8	10.2	86.3	3.5	63.8
Average		79.4	17.6 (4.2)	9.8	85.1	3.3	62.6
4µM	2000	78.8	21.3	9.3	71.3	2.8	58.8
	2001	76.3	23.8	9.4	73.8	2.5	56.3
Average		77.5	22.6 (4.8)	9.4	72.6	2.7	57.6
$LSD_{0.05}$[e]		ns[f]	0.5	1.3	4.9	0.6	6.2

[a] Contained thidiazuron (TDZ).

[b] Contained indole-3-butyric acid (IBA).

[c] Variance due to year and its interaction with pre-treatments were not significant.

[d] Between parenthesis are square root transformed values.

[e] To separate means of different concentrations of TDZ ($P < 0.05$) averaged over years

[f] Non-significant.

(Hartmann et al , 1997; Nehra and Stushnoff, 1989).

II-A-2. TDZ concentrations: Great number of shoots was produced from the SN explants incubated on the SRM with 2 μM /l TDZ (Table 2). The concentrations of TDZ higher than 2 μM did not increase the number of the harvested shoots per the nodal explant culture Less number of shoots was regenerated in the cultures of the SN on the medium with 1 μM TDZ than with 2 μM. Victor et al (1999), found that the supplements of TDZ resulted in an overall increase in the accumulation of the endogenous purine cytokinins. It is suggested, therefore, that the stimulation of shoot regeneration in vitro by TDZ is related to the endogenous level of the purine metabolites Optimum TDZ concentration differed. therefore, from plant species to another, according to the physiological status of the mother plants, the explant type and incubation condition of the culture. As low as 0.02 to 0.1 μM TDZ produced the greatest number of adventitious shoots and shoot buds in cultures of hypocotyl explants of sycamore maple (*Acer pseudoplatanus)* (Wilhelm, 1999). However, 20 μM TDZ was the most effective concentration in salad burnet (*Poterium sanguisorba* L.) (Babaoglu and Yorgancilar, 2000). In common lavender (*Lavandula vera* DC), 2 25 μM TDZ was found optimum (Andrade et al., 1999). The concentration higher than optimal TDZ or other purine cytokinins produced hyperhydricity in common lavender (Andrade et al., 1999). In the present study of salvia, on average, 13% of the cultures showed symptoms of vitrification on the medium with 1 μM TDZ (Table 2) The changes in the vitrification rate were not significant when the concentration of this cytokinin-like compound (TDZ) was elevated to 2 μM. However, further increase of the TDZ concentration significantly increased the vitrification rate. As high as 23% of the cultures showed vitrification in the existence of 4 μM TDZ (Table 2).

Another common adverse effect of high concentration of the cytokinins, in general. is the difficulty in rooting of the regenerated shoots (Khalafalla and Hattori, 2000; Tawfik and Noga, 2001). The inhibition of root formation on the TDZ-induced shoots is due to the increase of ethylene production (Khalafalla and Hattori, 2000). Use of 3 μM TDZ in salvia (Table 2) was shown to reduce the number of the formed roots per plantlet while 4 μM decreased both the percentage of rooted shoots and the number of the formed roots per plantlet. In particular, the decreased number of roots per plantlet seemed

to reduce the plantlet survival ex vitro (Table 2). Therefore, lower percentage of plants derived from shoots regenerated on the SRM with 3 or 4 μM TDZ survived during and after the acclimatization process than those obtained with 1 or 2 μM. The overall results presented here for the different tested concentration of TDZ suggest that 2 μM was the optimal level for plant regeneration of salvia from the split-nodal cultures.

II-B. Culture on medium with benzyladenine (BA) alone or plus TDZ (Expt. II)

Except for the number of shoots produced per explant, no significant differences were detected among the various BA, TDZ and BA plus TDZ supplements in the SRM (Fig. 3). More shoots per SN explant were obtained when 2 μM TDZ (reference treatment) was added into the SRM than using BA at concentrations of 1 or 2 or 3 μM (Fig. 3 C). The more effectiveness of TDZ for shoot regeneration in comparison with BA has been widely documented, for instance, in European beech (*Fagus sylvatica* L.) and Oriental beech (*F. Orientalis* Lipski) (Cuenca et al., 2000) and in *Rubus* (raspberry and blackberry) (Fiola et al., 1990). TDZ was reported to induce as much as 4 to 6 times the number of shoots produced per explant on medium with BA (Cuenca et al., 2000, Fiola et al., 1990; Sriskandarajah et al., 2001). The optimal effective level of TDZ is about one tenth the level of BA (Fiola et al., 1990). The data of the plant regeneration obtained in the present study, is on line with those reported. From different plant species however, these results are considered new in tissue culture of salvia.

The combination of 2 μM TDZ and 1 μM BA was similar to the use of sole 2 μM TDZ regarding the number of the harvested shoots per SN explant (Fig. 3C). However, supplements of 2 or 3 μM BA plus 2 μM TDZ increased the number of the regenerated shoots as compared to 2 μM TDZ alone. Combinations of TDZ and BA have been recently pointed out as a treatment for the most effective responses of shoot regeneration in vitro in a number of plant species. These included, for instance, faba bean (*Vicia faba* L.) (Khalafalla and Hattori, 1999) and green ash (*Fraxinus pennsylvanica* Marsh.) (Kim et al., 1997). BA may be involved in a complementary way to the action of TDZ (a substituted urea compound) for stimulating the accumulation of indogenous purine cytokinins.

There has been a great recent interest in exploiting the potential applications of cellular- and molecular-based biotechnology in the improvement of economically important plant species. Regeneration of plants from induced

adventitious meristems and shoots is essentially required to realize the potentiality of such applications of biotechnology. In this context, since the present study utilized wounded tissues of a pre-existing meristem and excluded an intermediate callus phase, it could be useful in both the clonal multiplication and the production of genetically modified salvia plants via *Agrobacterium*-mediated transformation. A combination of 2 µM of TDZ and BA is proposed for salvia regeneration from split-nodal explants.

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تكون المرستيمات العرضية ونمو الفروع الخضرية للسالفيا فى زراعات العقد المنشقة

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يعتبر الثيديزون (تى دى ذى) من المركبات أشباه السيتوكينينات والتى عادة ما تستخدم بتركيزات منخفضه لتحسين توالد النموات الخضرية وتكاثرها فى زراعات الأنسجه للعديد من الانواع النباتيه – وقد اشارت الدراسات الحديثة إلى أن توليفه من " تى دى ذى " والبنزيل أدنين " بى اه " أكثر تأثيرا من ال "تى دى ذى " منفردا كما أن معاملة الإظلام لمدة أسبوع قد حسنت من تكون المرستيمات العرضية للنموات الخضرية – وهناك حاجه لدراسة هذه التأثيرات فى زراعات الأنسجة للسالفيا والتى تعتبر من النوابل والنباتات الطبيه الهامة ولا يوجد طريقة صادقة لدفع النموات الخضرية لها كما أن إستجابتها للبنزيل أدنين ضعيفة. فى الدراسة الحالية تم أعداد الاجراء النباتيه من عقد الساق من نباتات تم إكثارها داخل الأنابيب ثم تم شق هذه العقد طوليا إلى نصفين مرورا بالبرعمين الخضريين الجانبيين بعد إزالتهما وخدش مكانهما ، ثم زرعت بعد ذلك على بيئه " ام اس " لموارشيج وسكوج (١٩٦٢). وشملت الدراسة تجربتين متتابعتين: فى التجربة الاولى تم زراعة العقد المنشقه على تلك البيئة الغذائيه بدون أو بعد إضافة واحد إلى ٤ ميكرومول من " تى دى ذى " وقد تم حفظ الزراعات إما تحت الإضاءة لمدة خمس أسابيع او لمدة اسبوع فى الإظلام تلاها ٤اسابيع تحت الضوء. حيث أظهرت النتائج أن معاملة الإظلام المبدئى زادت من عدد النموات الخضرية وكذلك كان تركيز ٢ ميكرومول من " تى دى ذى " هو التركيز الأمثل لتوالد النموات الخضرية وقد إتضح ذلك عندما زرعت على بيئة خالية من منظمات النمو لإستطالة الفروع. وفى التجربة الثانية أستخدمت البيئه محتوية إما على " تى دى ذى " منفردا (٢ ميكرومول) أو مضافا اليه بنزيل أدنين " بى ايه " بتركيز من واحد إلى ٣ ميكرومول بى ايه. وقد ظهر من النتائج أن اكبر عدد من النموات الخضرية كان عند إضافة ٢ ميكرومول " بى ايه " الى البيئه المحتويه على ٢ ميكرومول " تى دى ذى ". وفى كلا التجربتين كونت نسبة عالية من الفروع الخضرية جذورا عندما فصلت ونقلت على بيئة أخرى تحتوى على ٥ ميكرومول إندول بيوتريك اسيد (آى بى ايه).

<u>الفائدة التطبيقية للدراسة</u>:

حيث أن الدراسة الحالية تم فيها إستخدام أجزاء نباتيه بها مريستيمات سابقه الوجود بعد جرحها ، ولم يتم أنتاج النموات الخضرية من خلال طور وسطى لتكوين الكلس ، فان هذه الدراسة يمكن أن تكون مفيده فى كل من الأكثار الدقيق وفى أجراء التحول الوراثى باستخدام الأجروبكتريم فى السالفيا .