INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
11–679/ZIP/2012/14–3–445–449
http://www.fspublishers.org

Full Length Article

Callus Induction and Plant Regeneration from Mature Seeds of *Salvia splendens*

LIU HUII[1]†, ZHANG GUOPING, SHEN GUOZHENG, RUAN SONGLIN AND FU QIAOJUAN†
Agronomy Department, Zhejiang University, Hangzhou 310029, P.R. China
†*Department of Horticulture, Hangzhou Academy of Agricultural Sciences, Hangzhou 310024, P.R. China*
[1]Corresponding author's e-mails: zhanggp@zju.edu.cn; liuhui518lh@163.com

ABSTRACT

The effect of various combinations of plant growth regulators (PGRs) and explant types on the callus induction and plant regeneration of *Salvia splendens* Ker-Gawl was investigated. Results showed that the medium containing 6-benzylaminopurin (BAP), naphthalene acetic acid (NAA) and 2,4-Dichlorophenoxy acetic acid (2,4-D), could induce callus from stem and leaf explants; whereas, the medium containing only BAP and NAA failed to induce organogenic callus from mature seeds. For the two cultivars examined, Queen and vista, the highest rate of callus induction from leaf and stem explants occurred in the medium containing 1.0 mg L^{-1} 2,4-D and 1.5 mg L^{-1} BAP; whereas the highest rate of callus induction from mature seeds occurred in the medium containing 1.0 mg L^{-1} BAP and 2.0 or 2.5 mg L^{-1} 2,4-D. Although shoots were sporadically initiated at very low frequencies from the calli induced from stem segments with nodes, the calli induced from stem or leaf segments did not form shoots. Many regenerated plantlets were obtained from the cotyledon-derived calli, induced from mature seeds and the medium containing 2.0 mg L^{-1} 1-phenyl-3-(1,2,3-thia-diazol-5-YL) urea (TDZ) and 1.0 mg L^{-1} kinetin(KT), showed the highest efficiency in shoot regeneration of the two *S. splendens* cultivars. © 2012 Friends Science Publishers

Key Words: Mature seed; Callus; Regeneration; Plant growth regulator
Abbreviations: BAP, 6-benzylaminopurin; NAA, naphthalene acetic acid; 2,4-D, (2,4-Dichlorop- henoxy) acetic acid; TDZ, 1-Phenyl-3-(1,2,3-Thia-Diazol-5-YL) urea; KT, kinetin; MS, Murashige and Skoog; PGR, Plant Growth Regulator.

INTRODUCTION

Ornamental bedding plants include many annual and perennial plant species, which may provide seasonal colors to landscapes and home gardens (Wu *et al*., 2009). Among a wide variety of bedding plants, *Salvia splendens* Ker-Gawl of the Labiatae family is now most commonly used mainly due to their brilliant color during the warm season (Liu *et al*., 2011). However, *S. splendens* is quite limited in their color type, only to white, salmon, purple and the traditional fresh red. Moreover, the plants are relatively sensitive to high temperature. As an introduced plant, so far few heat-tolerant *S. splendens* cultivars or lines are present in China. Thus, the limited flower colors and weak heat-tolerance greatly affect the wide planting of *S. splendens* in China, particular in the areas with high temperature during summer. It is obvious that development of new *S. splendens* cultivars with other colors and heat tolerance is imperative for increasing their ornamental and economic values.

The rapid development of genetic engineering offers new and effective approaches for enriching colors and improving the abiotic resistance of plants, including *S. splendens*. Genetic transformation or transgenic technology has been widely used in angiosperm breeding, because it can alter the special traits of flowering plants faster and more effectively than conventional methods. However, the application of transgenic technology depends on a reliable plant regeneration system through callus induction. Callus proliferation with high regeneration capacity is necessary for production of transgenic *S. splendens* plants. However, there is no report up to date on plant regeneration of the flowering plant via *in vitro* callus culture from explants.

The present study was aimed at determining the effect of explant types (leaf blade, stem segments with or without nodes, and mature seeds) and plant growth regulators (PGRs) on the callus induction and shoot regeneration of *S. splendens*, and developing an efficient plant regeneration protocol, which could be used for molecular improvement of *S. splendens* plants.

MATERIALS AND METHODS

Experimental materials: Mature seeds of two *S. splendens* genotypes, Vista and Queen, were kindly provided by Zhejiang Hong Yue Flower Co. Ltd., China and used in the study. The seeds were sequentially surface sterilized with 75% (v/v) ethanol for 1 min and 0.1% mercuric chloride solution for 8 min, and then thoroughly rinsed four times with sterile distilled water. Finally, intact seeds were planted in PGR-free MS basal medium (Murashige & Skoog, 1962) containing 30 g L^{-1} sucrose, solidified in 7 g L^{-1} agar, and then the solution was adjusted to pH 5.8. The seeds were

To cite this paper: Huii, L., Z. Guoping, S. Guozheng, R. Songlin and F. Qiaojuan, 2012. Callus induction and plant regeneration from mature seeds of *Salvia splendens*. *Int. J. Agric. Biol*., 14: 445–449

cultured for one week in dark for germination and then in light under a 14-h photoperiod (5:00-19:00) with 200-300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (white light) light intensity at 25±2℃. In general, *S. splendens* seeds germinated and developed into seedlings high 3-5 cm after around 10 and 20 d, respectively.

Explants and cotyledons: Explants (leaf blade & stem segments with or without node) from different tissues were taken from 40-d-old aseptic plantlets cultured in MS basal medium. The leaves and stems were cut into small pieces, around 1 and 6-8 mm, respectively. All explants were placed in callus-inducing medium.

The sterilized seeds were sliced longitudinally into two halves, using sterilized forceps and a bistoury after cultured on MS basal medium in dark for 24 h (Fig. 1a). Then, seed coats were removed, and the two halves of each sliced seeds were plated on the callus-inducing medium, with the cut surface placed toward to the medium (Fig. 1b). Cotyledonary explants grown from the mature embryos after about 10 d were then cultured on the callus-inducing medium for callus induction (Fig. 1c).

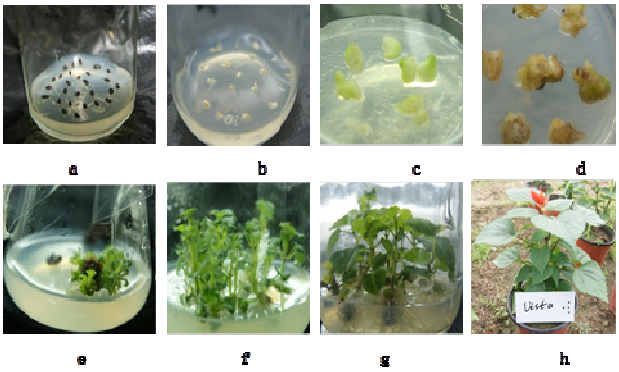
Callus induction: The basal medium for callus induction was ½ MS medium supplemented with different concentrations of 6-benzylaminopurin (BAP) or 2,4-dichlorophenoxyacetic acid (2,4-D) (0.5, 1.0, 1.5, 2.0, 2.5, or 3.0 mg L^{-1}) and 0.2 mg L^{-1} naphthalene acetic acid (NAA), or 2,4-D (0.5, 1.0, 1.5, 2.0, 2.5, or 3.0 mg L^{-1}) and BAP (1.0 or 1.5 mg L^{-1}). All explants were cultured on different induction media to determine the optimum medium for primary callus induction. The induction was carried out in continuous darkness at 25±2℃, and calli were sub-cultured on a fresh medium every 15 d.

The culture was routinely maintained in an original induction medium as soon as the organogenic callus was formed.

Plant regeneration: The basal media used in callus differentiation was the same as callus induction. The proliferated calli from cotyledons and explants after induction for 7-9 weeks were transferred to the regeneration medium supplemented with different concentrations of 2.0 mg/L BAP and 1.0 mg/L KT or 0.1 mg/L NAA, 2.0 mg/L TDZ and 1.0 mg/L KT or 0.1 mg/L NAA. The cultures were placed under light conditions (200-300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ illumination for 16 h photoperiod, 5:00-21:00) at 25±2℃. After 6-8 weeks, the number of adventitious shoots regenerated on each flask of each media type was recorded.

Regenerated shoots, when their heights reached 2–3 cm, were transferred for root development onto 1/2MS medium supplemented with 0.5 mg/L NAA, cultured under light conditions (200-300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ illumination for 16 h photoperiod) at 25±2℃. After 3-4 weeks, individual rooted plantlets 7–10 cm in length, were transferred directly from the culture room to a glasshouse, while the culture-flask covers remained closed. After one week, the flasks were opened and a little distilled water was added for acclimatization for 2-3 days. Then developed plantlets were gently washed to remove agar from their roots and were transferred to peat mixed with perlite (8:1) in plastic cups (upper diameter 6 cm × length 8 cm) and placed in a glasshouse. After two weeks, the plants were removed out of the glasshouse and placed in shade (approximately 60% shade) under natural conditions.

Fig. 1: Plant regeneration from mature seeds of *Salvia splendens*



All the media above mentioned contained 30 g/L sucrose, 0.5 g/L casein and were adjusted to pH 5.8, solidified with 7 g/L agar. PGRs were added to the medium before autoclaving. The media were autoclaved for 20 min at 121℃ and 1.1 kg/cm^2 pressure.

Statistical analysis: The experiment was laid in a completely randomized design with 10 culture-flasks (replicates) for each treatment. Six explants (leaf blade, stem segments or stem segments with node) or 10 half-seeds were plated in each flask. The experiment was conducted twice and data presented in tables were averages of the two experiments for each treatment. Analysis of variance (ANOVA) and mean separation were performed using least significant difference (LSD).

RESULTS

Effect of genotype and culture media on primary callus induction of *S. splendens*: The two cultivars examined were able to produce loose and translucent calli from assayed explants except for mature seeds within 7-9 weeks of incubation on the all induction medias (Table IA). As shown in Table IA, explants of stem segments and the segments with node of both *S. splendens* cultivars were easier to be induced into calli under lower BAP concentration, and the BAP concentration higher than 2.0 mg/L BAP could inhibit formation of callus. Concerning leaf blade, the culture media supplemented with 2.0 mg/L BAP and 0.2 mg/L NAA could induce formation of callus at rate of 90% for Queen and 80% for Vista. While the combination of 2, 4-D and NAA resulted in lower induction of calli for the two cultivars relative to the combination of BAP and NAA. From Table I, it could be also seen that on the medium supplemented with both BAP and NAA, callus could not be induced from seeds for any cultivar. However, when seeds were cut into two parts, their

Table I: Effects of various combinations of plant growth regulators at different concentrations on callus induction rate from explants of *Salvia splendens*[*]

(A)

Growth regulators (mg/L)		Callus initiation rate (%)							
		Queen				Vista			
BAP	NAA	Stem segments	Stem segment with node	Leaf segment	Seeds (cotyledons)	Stem segments	Stem segment with node	Leaf segment	Seeds (cotyledons)
0.5	0.2	100 a	100 a	51.5 e	0 g	100 a	100 a	52 e	0 f
1.0		100 a	100 a	82 b	0 g	96 ab	96 a	77.5 bc	0 f
1.5		100 a	96 b	80 b	0 g	94.5 b	85 b	83 a	0 f
2.0		91.5 b	90 c	90 a	0 g	90 c	85.5 b	80.5 ab	0 f
2.5		82.5 c	82.5 e	65.5 c	0 g	86 c	78 c	75 c	0 f
3.0		79 d	72 g	62 d	0 g	76 d	73.5 c	63 d	0 f
2,4-D									
0.5		90 b	87 d	67.5 c	20 f	100 a	97.5 a	65.5 d	34 e
1.0		71 e	83.5 e	31 f	42.5 e	72 d	77.5 c	42 f	54.5 d
1.5		51.5 h	74.5 f	20.5 gh	63 d	57 e	66 d	37 fg	71 c
2.0		50 h	66 h	21 g	78 b	53 ef	61 d	32.5 g	82 a
2.5		57.5 g	65 h	18 h	75.5 c	51.5 f	63 d	21 h	78 ab
3.0		60 f	58 i	31 f	80 a	51.5 f	61 d	23 h	74.5 bc

(B)

Growth regulators (mg/L)		Callus initiation rate (%)							
		Queen				Vista			
2,4-D	BAP	Stem segments	Stem segment with node	Leaf segment	Seeds (cotyledons)	Stem segments	Stem segment with node	Leaf segment	Seeds (cotyledons)
0.5	1.0	72 bc**	80 ab	72.5 ab	34.5 f	76 bc	83 ab	76.5 a	52 d
1.0		74.5 b	78.5 abc	65 c	46 e	82 a	85 a	70.5 ab	53.5 d
1.5		73.5 bc	72 cd	53.5 d	65 c	76.5 abc	78.5 ab	64 b	68 bc
2.0		72 bc	66 de	53 d	83 a	74 cd	67 cd	56.5 c	83.5 a
2.5		68.5 cd	73 bcd	51.5 d	80.5 ab	72 cd	64 d	49 de	86.5 a
3.0		52 e	63 e	42 e	74 b	61.5 e	66 d	43.5 e	73.5 b
0.5	1.5	71 bc	82 a	73 ab	42 e	70 d	75.5 b	71.5 a	51.5 d
1.0		83.5 a	84 a	75.5 a	57 d	81.5 ab	86.5 a	70 ab	63 c
1.5		72.5 bc	77 abc	68.5 bc	66.5 c	80.5 ab	82 ab	64 b	68 bc
2.0		65 d	71 cd	50.5 d	78 ab	71 cd	75 bc	53 cd	69 bc
2.5		64 d	72 cd	52 d	75 b	63 e	67 cd	42.5 e	71 bc
3.0		57 e	65.5 de	43 e	76.5 ab	51.5 f	62.5 d	35 f	71.5 bc

*Ten culture flasks (replicates) for each treatment. Six explants (leaf blade, stem segments or stem segments with node) or ten half-seeds were plated in each flask. The experiment was conducted twice and data presented in tables were averages of the two experiments for each treatment

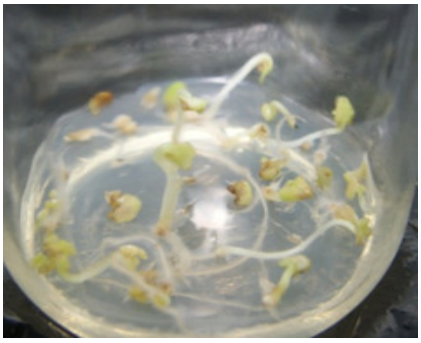
The percentage of callus induction was determined at the 40 day after culture and was calculated as (number of explants forming callus /number of all uncontaminated explants plated on initiation medium)×100

**Values followed by the same letter are not significantly different at $P = 0.05$ according to the ANOVA test

cotyledonary explants from mature embryo became swelled and germinated after about one week incubation and developed into seedlings (Fig. 2). By contrary, on the medium supplemented with both 2, 4-D and 0.2 mg/L NAA, callus could be induced from the cotyledons for both cultivars. Moreover, higher 2, 4-D concentration would promote the induction of organogenic callus, although there was no significant difference on the callus induction between 2.0 and 3.0 mg/L 2, 4-D.

There were obvious differences in the properties of induced calli from all explants among the media with different PGR addition. The calli induced on the medium supplemented with BAP were loose, easily became albino friable or brown, leading to death after long-time subculture (results not shown). However, the induced rate was much higher on the medium supplemented with BAP than the BAP-free medium. On the other hand, the medium with addition of 2, 4-D induced the compact and green calli, which showed low occurrence in becoming brown under long-time subculture. In the current study, the effect of various combinations of BAP and 2,4-D concentration on callus induction rate was also investigated, in order to determine the optimal PGR application in inducing callus of *S. splendens*. It can be seen from (Table IB) that on the all

Fig. 2: The cotyledonary explants from mature embryos bulged and then germinated after about 1 week incubation on the media supplemented with BAP and NAA



media supplemented with BAP and 2,4-D, all explants and cotyledons of both cultivars can be induced high-quality calli after at least four weeks culture. Moreover, obvious difference could be found in the induction efficiency among the media containing 2,4-D of 0.5-3.0 mg/L. On the medium supplemented with both 1.5 mg/L BAP and 1.0 mg/L 2,4-D, the highest rate of callus induction was detected from all kinds of explants for Queen, and the same was true for Vista. On the whole, the stem segments or stem segments with node are easier to be induced formation of callus than leaf segments. In addition, the medium supplemented with both 1.0 mg/L BAP and 2.0 or 2.5 mg/L 2,4-D had the highest induction rate of calli from the cotyledons for any cultivar.

Proliferation of primary callus and shoot regeneration: In order to obtain enough compact and green calli for plant regeneration, the callus induced from explants were transferred onto the fresh media supplemented with 1.5 mg/L BAP and 1.0 mg/L 2,4-D for proliferation, while the callus induced from the cotyledons were further cultured on the medium containing 1.0 mg/L BAP and 2.0 mg/L 2,4-D. These calli were sub-cultured routinely every two weeks onto the fresh medium with the same chemical composition under darkness at 25±2℃. The morphology of calli was observed at 3-day interval. Only the callus with light green color, nodular structure, and irregular appearance were used for shoot induction. After two sub-culture cycles, proliferated callus were separately transferred to regeneration medium supplemented with PGRs. Our results showed that after 6-week sub-culture on the regeneration medium, the calli induced from leaf blade and stem segments of the two cultivars became watery and finally turned into brown, losing the capacity of differentiation. As a result, no adventitious shoots were induced from the calli on the four kinds of differentiation media (Table II), suggesting it is quite difficult for the callus induced from stem or leaf blade to develop into plantlet. On the other hand, the calli induced from stem segments with node and cotyledons became compact, friable, irregular in shape and light green (Fig. 1D), could further differentiate into shoots via organogenesis (Fig. 1E). As a result, shoots could be observed in such organogenic calli on the four differentiation medium (Table II). However, only a part of such calli could regenerate shoots, the calli induced from stem segments with node was very low in regenerating rate, with the highest rate being less than 20% for the two cultivars (Table II). It was found that the cotyledon-derived calli from mature seeds had the high capacity of forming shoot via organogenesis. Among the four differentiation media, the medium containing 2.0 mg/L TDZ and 1.0 mg/L KT had the highest shoot regenerating rate, 70% for Queen and 72.5% for Vista, respectively (Table II). Obviously, it is possible to obtain a lot of regenerated plantlets of *S. splendens* using cotyledon-derived calli from mature seeds (Fig. 1).

a: sterilized seeds cultured for 24 h in MS basal medium under dark, b: sterilized seeds were sliced longitudinally into two halves, which were plated on the callus induction media with seed coats removed, c: the cotyledonary explants grown from mature embryos in about 10 days, d: organogenic light green calli induced from cotyledons after one subculture, e: multiple shoots regenerated from cotyledon-derived calli, f: regenerated plantlets were transferred for root development, g: regenerated plantlets with healthy root grown in culture room, h: regenerated *S. splendens* plants normally grown in the field under natural conditions.

Plant regeneration and transplant: It was found in the experiment that when the calli with multiple shoots were cultured on the regeneration medium for more than eight weeks, they would gradually become brownish and the regenerated shoots turned to withering. Hence the multiple shoots should be excised in time and transferred onto a fresh medium for proliferation and growth. When the regenerated plantlets were 2–3 cm in height, they were transferred onto rooting medium for root development. After about four weeks, around 85% of regenerated plantlets initiated roots (Fig. 1F). When the plantlets were 7-10 cm in height and well-rooted, they could be transplanted into pots containing peat and perlite (volume 8:1) and placed in a greenhouse. After two weeks the plants could be moved out of the greenhouse and allowed to grow in a shaded (approximately 60% shade) place under natural conditions. In our experiment, the survival rate of transplanted plantlets was up to 70%, and all regenerated-plantlets appeared to be normal in phenotype (Fig. 1H).

DISCUSSION

It was reported that some salvia plants, such as *S. miltiorrhiza* (Shimomura *et al*., 1991), *S. sclarea* (Liu *et al*., 2000) and *S. officinalis* (Kintzios *et al.*, 1999) could develop into cluster shoots from callus. However, no regenerated plantlet has been obtained from the calli induced from explants including shoot apex and leaf blade for *S. splendens* (Li *et al*., 2005). In the present study, influence of different PGR combinations on the induction of callus and plant regeneration from various explants of *S. splendens*, including leaf and stem segments, and mature seeds was investigated. The results showed that all media containing the combinations of three PGRs i.e., BAP, NAA and 2,4-D could induce callus from leaf and stem segments (Table I), while the combination of BAP and NAA could not induce formation of callus from mature seeds, suggesting that 2,4-D is crucial to the formation of callus from mature seeds (Vikrant *et al*., 2003). The similar finding was observed in ash gourd (Thomas *et al*., 2004). By contrast, the highest rate of callus induction from explants of the stem and leaf segments occurred on the medium containing 1.0 mg/L 2, 4-D and 1.5 mg/L BAP and from mature seeds occurred on the medium supplemented both 1.0 mg/L BAP and 2.0 or 2.5 mg/L 2, 4-D. Shoots were initiated at very low frequency from calli induced from stem segments with node, while no shoots were obtained from the calli induced from stem or

leaf segments (Table II). However, the cotyledon-derived organogenic calli from mature seeds were directly capable of forming shoot primordial via organogenesis. Thus, a lot of regenerated plantlets of the two *S. splendens* cultivars were obtained, and the medium containing 2.0 mg/L TDZ and 1.0 mg/L KT showed the highest efficiency in terms of shoot differentiation. As far as could be ascertained, it is the first report that regenerated plantlets of *S. splendens* are obtained from the organogenic calli induced from mature seeds.

The explants of mature seeds have been widely used in tissue culture (Özgen *et al*., 1998; Delporte *et al*., 2001). The efficient *in vitro* regenerating systems of inducing callus from mature seeds have been developed in many plants, such as wheat (Chauhan *et al*., 2007), barley (Özgen *et al*., 2007; Gürel *et al*., 2009), rice (Lee *et al*., 2002; Carson*o et al.,* 2006), maize (Huang *et al*., 2004) and ash gourd (Thomas *et al*., 2004). Mature embryos from dry seeds, used as the explants, have several advantages: easy to be handled, available in the whole year, and sufficient in quantity. In the current experiment, the cotyledonary explants grown from mature embryos cultures, and then were successfully induced into primary calli, which could develop into organogenic calli and such calli further differentiated into multiple shoots on the regeneration medium via organogenesis (Fig. 1E).

It is reported that the brown change of callus is a bottleneck for success of tissue culture. In our study, the calli induced from cotyledons became also gradually brown in long-time sub-culture even 0.2 g/L Vc or 20 g/L mannitol was added in medium (data not shown). Previous studies suggested that the brown change of callus is affected by many factors, including genotype, composition of medium, hormone and its concentration, and culture condition (Mayer, 1986; Hisaminato *et al.,* 2001; Shanjani, 2003). More studies are required to find out important factors which affect brown change of callus, in order to optimize the regenerating system of *S. splendens*. In addition, obvious difference was detected among the culture media in their effect on callus initiation of mature seeds and subsequent plantlet regeneration, but there was no distinct difference between the two *S. splendens* cultivars in the rate of regenerated plantlets. It may be suggested that the kind and concentration of hormones should be highlighted for further improving regeneration efficiency of *S. splendens*.

It is worth noting that mature seeds of *S. splendens* are available throughout the year, and suitable for use in tissue culture. As Fig. 1 showed that we developed a regenerating system of *S. splendens* using mature seeds as explants, and obtained the whole regenerated plants. It may be concluded that the developed system will be used in genetic transformation and molecular breeding of *S. splendens.*

Acknowledgement: This research was financed by Hangzhou Municipal Science and Technology Bureau and Zhejiang Provincial Natural Science Foundation of China.

REFERENCES

Carsono, N. and T. Yoshida, 2006. Identification of callus induction potential of 15 Indonesian rice genotypes. *Plant Prod. Sci.,* 9: 65–70

Chauhan, H., S.A. Desai and P. Khurana, 2007. Comparative analysis of the differential regeneration response of various genotypes of *Triticum aestivum*, *Triticum durum* and *Triticum dicoccum*. *Plant Cell Tiss. Org. Cult*., 91: 191–199

Delporte, F., O. Mostade and J.M. Jacquemin, 2001. Plant regeneration through callus initiation from thin mature embryo fragments of wheat. *Plant Cell Tiss. Org. Cult*., 67: 73–80

Gürel, F., S.Ö. Karaka, G. Albayrak and S. Air, 2009. Regeneration capacity of mature embryo-derived callus in barley (*Hordeum vulgare* L.). *Acta Biol. Hungarica*, 60: 309–319

Hisaminato, H., M. Murata and S. Homma, 2001. Relationship between the enzymatic browning and phenylalanine ammonia-lyase activity of cut lettuce, and the prevention of browning by inhibitors of polyphenol biosynthesis. *Biosci. Biotechnol. Biochem.*, 65: 1016–1021

Huang, X.Q. and Z.M. Wei, 2004. High-frequency plant regeneration through callus initiation from mature embryos of maize (*Zea may*s L.). *Plant Cell Rep.,* 22: 793–800

Kintzios, S., A. Nikolaou and M. Skoula, 1999. Somatic embryogenesis and *in vitro* rosmarinic acid accumulation in *Salvia officinalis* and *S. fruticosa* leaf callus cultures. *Plant Cell Rep.*, 18: 462–466

Lee, K., H. Jeon and M. Kim, 2002. Optimization of a mature embryo-based *in vitro* culture system for high-frequency somatic embryogenic callus induction and plant regeneration from *japonica* rice cultivars. *Plant Cell Tiss. Org. Cult*., 71: 237–244

Li, F.L., G.F. Hu and B.Z. Hu, 2005. Study on tissue culture and plant regeneration of the inflorescence in eight kinds color of *Saliva splendens. Biotechnology*, 15: 71–73

Liu, W.N., C.E. Chilcott, R.C. Reich and G.M. Hellmann, 2000. Regeneration of *Saliva sclarea* via organogenesis. *In Vitro Cellular Develop. Biol. Plant.*, 36: 201–206

Liu, H., G.P. Zhang, G.Z. Shen, Q.Q. Fu and S.L. Ruan, 2011. Responses of *Salvia splendens* cultivars 'Shenzhouhong' and 'Emperor' to thermo-stress. *J. Zhejiang Univ. (Agric. Life Sci.)*, 37: 155–161

Mayer, A.M., 1986. Polyphenol oxidases in plant-recent progress. *Photochemistry*, 26: 11–20

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant*, 15: 473–477

Özgen, M., M. Türet, S. Altnok and C. Sancak, 1998. Efficient callus induction and plant regeneration from mature embryo culture of winter wheat (*Triticum aestivum* L.) genotypes. *Plant Cell Reports*, 18: 331–335

Özgen, M., M. Yildiz, N. Koyuncu and S. Önde, 2007. The effect of seed size on tissue culture response of callus from endosperm-supported mature embryos of barley (*Hordeum vulgare* L). *Cer. Res. Commun.*, 35: 1415–1425

Shanjani, P.S., 2003. Nitrogen effect on callus induction and plant regeneration of *Juniperus excelsa. Int. J. Agric. Biol*., 5: 419–422

Shimomura, K., T. Kitazawa, N. Okamura and A. Yagi, 1991. Tanshinone production in adventitious roots and regenerates of *Salvia miltiorrhiza*. *J. Nat. Prod.*, 54: 1583–1587

Thomas, T.D. and K.R. Sreejesh, 2004. Callus induction and plant regeneration from cotyledoary explants of ash gourd (*Benincasa hispida* L.). *Sci. Hortic.*, 100: 359–367

Vikrant, R.A., 2003. Somatic embryogenesis or shoot formation following high 2,4-D pulse-treatment of mature embryos of *Paspalum scrobiculatum. Biol. Plant.,* 46: 297–300

Wu, D.L., S.W. Hou, P.P. Qian, L.D. Sun, Y.C. Zhang and W.J. Li, 2009. Flower color chimera and abnormal leaf mutants induced by $12C^{6+}$ heavy ions in *Salvia splendens* Ker-Gawl. *Sci. Hortic.*, 121: 462–467

(Received 02 December 2011; Accepted 04 February 2012)